TARCILLA CORRENTE BORGHESAN

# Diversidade e Filogenia de tripanossomatídeos parasitas de dípteros

Tese apresentada ao Programa de Pós-Graduação em Biologia da Relação Patógeno-Hospedeiro do Instituto de Ciências Biomédicas da Universidade de São Paulo, para obtenção do Título de Doutor em Ciências.

São Paulo
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Área de Concentração: Biologia da Relação Patógeno-Hospedeiro.

Orientadora: Profa. Dra. Marta Maria Geraldes Teixeira

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# CERTIFICADO DE ISENÇÃO

Certificamos que o Protocolo CEP-ICB Nº **591/13** referente ao projeto intitulado: "*Diversidade e Filogenia de tripanossomatídeos parasitas de moscas*" sob a responsabilidade de **Tarcilla Corrente Borghesan,** foi analisado na presente data pela CEUA - COMISSÃO DE ÉTICA NO USO DE ANIMAIS e pela CEPSH- COMISSÃO DE ÉTICA EM PESQUISA COM SERES HUMANOS, tendo sido deliberado que o referido projeto não utilizará animais que estejam sob a égide da lei 11.794 de 8 de outubro de 2008, nem envolverá procedimentos regulados pela Resolução CONEP nº196 de 1996.

São Paulo, 26 de abril de 2013.

PROF. DR. WOTHAN TAVARES DE LIMA
Coordenador da CEUA - ICB/USP

PROF. DR. PAOLO M.A ZANOTTO
Coordenador da CEPsh - ICB/USP

*Aos meus pais, Divina e Valdir pelo amor, carinho dedidados e pelos ensinamentos de vida que me fortalecem todos os dias. Aos meus irmãos Eveline e Valdir Filho pelo exemplo de compaheirismo. Ao Eder Gatti por todo o amor, companheirismo, compreensão presentes em todos os momentos...*

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## Prefácio

"Esta tese foi elaborada de acordo as normas da CPG/ICB relativas a outras formas de elaboração de tese de doutorado que permite a inclusão, como Anexos, de resultados já publicados ou submetidos em periódicos internacionais indexados em língua inglesa. Permite ainda que detalhes metodológicos e resultados obtidos sejam aqueles contidos nos artigos anexados ao corpo da tese."

**Anexo 1-** Phylogenetic Validation of the Genera *Angomonas* and *Strigomonas* of Trypanosomatids Harboring Bacterial Endosymbionts with the Description of New Species of Trypanosomatids and of Proteobacterial Symbionts

**Anexo 2-** Insect parasites of the genus *Angomonas*: Phylogeographical evidence of cryptic diversity, parasite-symbiont co-divergence and dispersion by blowflies throughout the Neotropics and Afrotropics

**Anexo 3-** Molecular Phylogenetic Redefinition of *Herpetomonas* (Kinetoplastea, Trypanosomatidae), a Genus of Insect Parasites Associated with Flies

## RESUMO

Borghesan TC. Diversidade e Filogenia de tripanossomatídeos parasitas de dípteros [tese (Doutorado em Biologia da Relação Patógeno-Hospedeiro)]. São Paulo: Instituto de Ciências Biomédicas, Universidade de São Paulo; 2013.

A família Trypanosomatidae compreende parasitas obrigatórios de invertebrados, principalmente insetos, plantas e de todas as classes de vertebrados, incluindo o homem. Tripanossomatídeos exclusivos de insetos, principalmente dípteros e hemípteros, foram descritos em mais de 400 espécies em todo o mundo. Os tripanossomatídeos de insetos são distribuídos nos gêneros *Crithidia*, *Blastocrithidia*, *Leptomonas*, *Herpetomonas* e *Wallaceina*. Esses gêneros não foram corroborados por análises filogenéticas e estudos mais abrangentes são necessários para uma análise contemporânea da taxonomia desses tripanossomatídeos. Os principais objetivos deste estudo foram rever a taxonomia do gênero *Herpetomonas* e dos tripanossomatídeos de insetos que abrigam simbiontes (SHT), que são dois grupos de parasitas comuns em dípteros. Para avaliar a diversidade genética e selecionar isolados para inclusão em árvores filogenéticas cerca de 130 tripanossomatídeos de moscas capturadas na America do Sul (Brasil, Venezuela e Colombia) e Africa (Moçambique, Guiné Bissau e Madagascar) foram comparados (V7V8 SSU rRNA – *DNA barcoding*) nesse estudo.

Para o primeiro estudo de SHTs, 4 espécies de referência e nove novos isolados foram incluídos em filogenias (SSU rRNA e gGAPDH) que apoiaram a monofilia de todos os SHTs e sua partição em dois subclados correspondentes aos gêneros *Strigomonas* e *Angomonas*. Estes gêneros foram posicionados distantes de *Blastocrithidia*, *Herpetomonas* e *Crithidia*. Dados morfológicos e análises filogenéticas foram empregados para a revalidação de *Angomonas,* contendo 10 flagelados incluindo *A. deanei* e *A. desouzai* e a nova espécie *Angomonas ambiguus*, e *Strigomonas* incluindo *S. oncopelti*, *S. culicis* e a nova espécie *Strigomonas galati*. As análises de 16S rRNA e ITS1rDNA revelaram três novas espécies de endossimbiontes (TPE- *trypanosomatid proteobacterial endosymbiont*), com congruência de filogenias inferidas apoiando evolução por co-divergência para a maioria das espécies dos tripanossomatídeos hospedeiros e seus simbiontes, com exceção de *A. ambiguus* que compartilha o simbionte com *A. deanei*.

A comparação (*DNA barcoding*) de 364 culturas de moscas coletadas na América do Sul e África permitiu identificar 70 culturas (20%) do gênero *Angomonas*, 50 (71%) delas de moscas da família Calliphoridae. Análises realizadas por PCR de amostras de tubo digestivo de moscas revelaram ~51% das moscas infectadas por *Angomonas*. Das 83 caracterizadas, 70 de cultura e 13 não cultivadas, 61 foram identificadas como *A. deanei*, 16 como *A. desouzai* e 6 como *A. ambiguu*s. Análises filogenéticas (gGAPDH) dos SHTs corroborou três clados principais liderados por *A. deanei*, *A. desouzai* e *A. ambiguus,* e revelou uma diversidade intra-específica representada por quatro genótipos de *A. deanei* e dois de *A. desouzai*. As filogenias dos SHTs e TPEs foram congruentes, com 4 genótipos de *A. deanei* congruentes com 4 de "*Ca*. K. crithidii" e dois de *A. desouzai* congruentes com dois de "*Ca.* K. desouzaii" corroborando a co-divergência dos SHTs e seus respectivos TPEs. *A. deanei* e *A. ambiguus* compartilharam o mesmo TPE. A congruência parcial entre SHTs e TPEs sugere uma predominância de propagação clonal com raros eventos de recombinação. Moscas dos gêneros *Chrysomya* e *Lucilia* (Calliphoridae) originaram 73% dos isolados, seguido por Muscidae com 14%. Apesar da forte

associação entre *Angomonas* e califorídeas, essas moscas também são frequentes hospedeiras de tripanossomatídeos de outros gêneros. Os principais hospedeiros de *Angomonas* são moscas do gênero *Chrysomya* e *Lucilia* (51%), ambos originários do Velho Mundo, enquanto *Cochliomya*, nativo da região neotropical, apresentou menores taxas de infecção (33%). A análise da associação *Angomonas*-TPE-califorídeos permitiu formular a hipótese de que a recente dispersão dessas moscas desempenhou um papel importante na dispersão de *Angomonas* no Neotrópico e na distribuição mundial das espécies/genótipos de *Angomonas*.

O gênero *Herpetomonas* foi criado para acomodar flagelados com formas opistomastigotas. No entanto, este parâmetro mostrou-se insuficiente levando a classificação de *Herpetomonas* como *Leptomonas* e *Phytomonas* ou a classificação como *Herpetomonas* de flagelados de outros gêneros. Para rever a taxonomia de *Herpetomonas* com base em análises filogenéticas, foram codificados (*DNA barcoding*) 527 tripanossomatídeos de insetos e 52 foram selecionados para inferências filogenéticas (gGAPDH e SSU rRNA). Os flagelados formaram um clado correspondente ao gênero *Herpetomonas*, que agora compreende 13 espécies válidas, 6 já conhecidas e 5 novas espécies. Estruturas primárias e secundárias de ITSrDNA foram comparadas para avaliação da variabilidade inter- e intra-específica. Microscopias de luz e eletrônica revelaram grande polimorfismo de *Herpetomonas*. Os resultados mostraram que algumas espécies de *Herpetomonas* são parasitas generalistas de moscas e parecem ser tão cosmopolitas quanto seus hospedeiros.
Os táxons que definimos nesse estudo podem ser revistos dependendo de novas evidências resultantes da análise de amostras adicionais utilizando novos marcadores moleculares. O aperfeiçoamento gradual das classificações é parte da dinâmica da taxonomia. Esperamos que os táxons propostos contribuam para a elaboração de critérios mais eficientes para uma melhor e mais natural classificação das espécies da família Trypanosomatidae.

**Palavras-chave:** Tripanossomatídeos, Filogenia, Taxonomia, Evolução, Diptera, Calliphoridae

## ABSTRACT

Borghesan TC. Diversity and Phylogeny of trypanosomatids parasites of dipteran [Ph. D. thesis (Biology of Host-Pathogen Interactions.)]. São Paulo: Instituto de Ciências Biomédicas, Universidade de São Paulo; 2013.

The family Trypanosomatidae comprises obligate parasites of invertebrates, mainly insects, of plants and of all classes of vertebrates including man. Trypanosomatids exclusive of insects have been reported in more than 400 species worldwide, mostly of dipterans and hemipterans. Insect trypanosomatids were distributed in the genera *Crithidia, Blastocrithidia, Leptomonas, Herpetomonas* and *Wallaceina*. These genera have not been corroborated by phylogenetic studies and broader analyses are required for a contemporary appraisal of the Taxonomy of insect trypanosomatids. Our main goals were to revise the taxonomy of the genus *Herpetomonas* and of Symbiont Harboring Trypanosomatids (SHTs), which are common parasites of dipterans. For this purpose, during our study we compared ~130 trypanosomatids from flies captured in South America (Brazil, Venezuela and Colombia) and Africa (Mozambique, Guinea Bissau and Madagascar).

We compared the barcodes (V7V8 SSU rRNA sequences) of 4 reference species and 9 new SHTs to assess the genetic diversity and to select isolates for inclusion in phylogenetic trees. The resulting phylogenies (SSU rRNA and gGAPDH) supported the monophyly of the SHT and its partition in two subclades corresponding to the genera *Strigomonas* and *Angomonas* revalidated in this study; these genera were distantly related to *Blastocrithidia*, *Herpetomonas* and *Crithidia*. Morphological and phylogenetic data enabled the description of *Angomonas* comprising 10 flagellates including *A. deanei* and *A. desouzai* plus the new *A. ambiguus,* and *Strigomonas* including *S. oncopelti, S. culicis* and the new *S. galati*. Analyses of 16SrRNA and ITS1rDNA revealed three new species of trypanosomatid proteobacterial endosymbionts (TPEs), and the partial congruence of SHT and TPE phylogenies support a co-divergent host-symbiont evolutionary history for most species excepting for *A. ambiguus*.

Barcoding of 364 cultures from flies collected in South America and Africa identified 70 cultures (20%) as *Angomonas,* 50 (71%) of them from blowflies (Calliphoridae). Surveys by PCR directly of 13 fly guts revealed that ~50% of the blowflies were infected by *Angomonas.* Of the 83 samples, 70 from cultures and 13 uncultivated, 61 were *A. deanei,* 16 *A. desouzai* and 6 *A. ambiguus.* Phylogenetic analysis (gGAPDH) of SHTs corroborated the existence of 3 main clades leaded by *A. deanei, A. desouzai* and *A. ambiguus,* and disclosed cryptic intra-specific diversity represented by four genotypes of *A. deanei* and two of *A. desouzai*. The phylogeny of the SHTs and TPEs were congruent. Four genotypes of *A. deanei* and "*Ca*. K. crithidii" and two genotypes of *A. desouzai* and "*Ca*. K. desouzaii" corroborated the SHT-TPE co-divergence. *A. deanei* and *A. ambiguus* shared the same TPE. The partial congruence between SHTs and TPEs suggests a predominant clonal propagation with sporadic recombination.

*Chrysomya* and *Lucilia* (Calliphoridae) yielded 73% of the isolates followed by Muscidae with 14%. Despite the strong *Angomonas*-blowflies association, these flies are also common hosts of trypanosomatids of other genera. The main hosts of *Angomonas* were blowflies of the genera *Chrysomya* and *Lucilia,* both originally from the Old World, while *Cochliomya*, native of the Neotropics, showed low infection rates

among the blowflies. The analysis of the tripartite association *Angomonas*-TPE-calliphorids allowed to hypothesize that the recent dispersion of blowflies played an important role in the dispersal of *Angomonas* into the Neotropics, and in the worldwide distribution of the species/genotypes of *Angomonas.*

The genus *Herpetomonas* was created to accommodate flagellates showing opisthomastigote forms. However, this parameter proved insufficient permitting the misplacement of *Herpetomonas* within *Leptomonas* and *Phytomonas* or the classification as *Herpetomonas* of flagellates of other genera. To review the taxonomy of the genus *Herpetomonas* through phylogenetic analyses we analyzed the barcodes of 527 insect trypanosomatids and selected 52 for gGAPDH and SSU rRNA phylogenetic inferences. The selected flagellates clustered into a monophyletic assemblage corresponding to the genus *Herpetomonas* that now comprises 13 valid species, 6 already known and 5 new species. ITS1 rDNA primary and putative secondary structures were compared for evaluation of inter- and intraspecific variability. Light and electron microscopy revealed an extreme polymorphism within *Herpetomonas*. Our findings showed that some species of *Herpetomonas* are generalist parasites of flies and appear to be as cosmopolitan as their hosts.

The taxa we have nominated can be reviewed at any time in the presence of new evidence from the analysis of additional samples using new molecular markers. The gradual improvement of classifications is part of the dynamics of taxonomy. We hope that the proposed taxa may help to elaborate more efficient criteria for a better and more natural classification of the Trypanosomatidae.

**Keywords:** Trypanosomatids, Phylogeny, Taxonomy, Evolution, Diptera, Calliphoridae

## LISTA DE ABREVIATURAS E SIGLAS

| | |
|---|---|
| B | Bayesiana |
| BAB | Meio agar sangue (“ Blood Agar Base”) |
| cGAPDH | Gliceraldeído 3-fosfato dehigrogenase citosólica |
| CHCl3 | Clorofórmio |
| DMSO | Dimetilfulfóxido |
| dNTP | Desoxirribonucleotídeos Fosfatados |
| DNA | Ácido desoxiribonucleico |
| EDTA | Ácido etileno diamino tetracético |
| g | grama |
| gGAPDH | Gliceraldeído 3-fosfato dehigrogenase glicossomal |
| KCl | Cloreto de Potássio |
| kDNA | “Kinetoplast desoxiribonucleic acid” |
| $KH_2PO_4$ | Fosfato de Potássio |
| HCl | Ácido clorídrico |
| IGS | “Intergenic spacer” |
| ISC | Insect sample collectio |
| ITS | “Internal trascribed spacer” |
| Kb | Kilobases |
| LB | “Luria-Bertani media” |
| LIT | Meio de cultura Infuso de fígado |
| LSU | “Large subunit” |
| M | Molar |
| mg | Miligrama |
| $MgCl_2$ | Cloreto de magnésio |
| min | Minutos |
| ml | Mililitro |
| mM | Milimolar |
| MP | Máxima Parcimônia |
| MV | Máxima Verossimilhança |
| mya | “millions years ago” |
| NaCl | Cloreto de sódio |
| NADH | Nicotinaanida adenine dinocleótido hidreto |
| $Na_2HPO_4$ | Fostato dissódico |
| NaOH | Hidroxido de sódio |
| ng | nanograma |
| pb | Pares de bases |
| PBS | Salina em tampão fosfato |
| PCR | Reação em cadeia da polimerase |
| RNA | Ácido ribonucleic |
| RNAse | Ribonuclease |
| rDNA | ácido desoxiribonucléico |
| rRNA | Ácido ribonucléico ribossômico |
| Sarkosil | Lauril sarcosinato de sódio |
| SE | Solução salina Tris-EDTA |
| SFB | Soro fetal bovino |
| TAE | Tampão Tris acetato-EDTA |
| TE | Tampão tris-EDTA |

| | |
|---|---|
| UV | Luz ultravioleta |
| TCC | Tripanosomatid culture collection |

**SUMÁRIO**

# 1. INTRODUÇÃO

## 1.1 A família Trypanosomatidae

A família Trypanosomatidae é formada por eucariotos unicelulares e uniflagelados pertencentes à classe Kinetoplastea que, juntamente com os euglenídeos e os diplonemídeos formam o filo Euglenozoa (Eukaryota: Excavata). Os estudos filogenéticos mais recentes do filo Euglenozoa apoiam a hipótese de que os cinetoplastídeos são mais relacionados com os diplonemídeos (Breglia et al., 2007, Busse e Preisfeld, 2002; 2003; Hughes e Piontkivska, 2003; Roger e Simpson, 2009; Simpson et al., 2006). A classe Kinetoplastea, originalmente formada pelas subordens Trypanosomatina e Bodonina, ambas definidas segundo parâmetros morfológicos, caracteriza-se pela presença do cinetoplasto, uma região especializada da mitocôndria composta por moléculas circulares de DNA (kDNA) concatenadas em uma rede única (Simpson et al., 2006; Stevens, 2008). Estudos filogenéticos baseados em marcadores moleculares levaram a uma revisão taxonômica do grupo, propondo a separação da classe Kinetoplastea em duas subclasses: Prokinetoplastina e Metakinetoplastina. A subclasse Prokinetoplastina possui uma única ordem (Prokinetoplastida) formada por *Ichthyobodo necator,* uma espécie ectoparasita de peixes, e *Perkinsiella amoebae*-like, um endossimbionte de amebas do gênero *Neoparamoeba*. A subclasse Metakinetoplastina inclui quatro ordens, sendo três de bodonídeos (Neobodonida, Parabodonida e Eubodonida), que incluem espécies de vida livre e parasitárias, e a ordem Trypanosomatida que alberga uma única família (Trypanosomatidae) de espécies de parasitas obrigatórios (Moreira et al., 2004). Estudos filogenéticos empregando genes codificantes de proteínas (Simpson e Roger, 2004) e análises filogenômicas (Deschamps et al., 2011) apóiam a hipótese de que os tripanossomatídeos posicionam-se como um grupo apical dentro da irradiação dos Metakinetoplastina e tem como grupo irmão Eubodonida, tornando, assim, os bodonídeos parafiléticos. Os protozoários da família Trypanosomatidae apresentam ampla distribuição geográfica e parasitam todas as classes de vertebrados, invertebrados (especialmente Diptera e Hemiptera) e plantas. Embora a maioria dos representantes dessa família não seja patogênica para seus hospedeiros, alguns são importantes agentes etiológicos de doenças

humanas e de animais domésticos, como as tripanossomíases Africana e Americana e as leishmanioses (Hoare e Wallace, 1966; Simpson et al., 2006; Vickerman, 1976).

Tradicionalmente, a taxonomia da família Trypanosomatidae tem sido baseada em parâmetros morfológicos, ciclo de vida e hospedeiros de origem. Quanto ao ciclo de vida, os tripanossomatídeos podem ser heteroxênicos, quando participam do seu ciclo biológico dois hospedeiros, sendo um invertebrado e um vertebrado, ou monoxênicos, quando completam seu ciclo de vida em apenas um hospedeiro, geralmente insetos de diversas ordens (Simpson et al., 2006; Stevens, 2008, Vickerman, 1976, Wallace, 1966; Wallace et al., 1983). Os tripanossomatídeos diferem quanto à forma, comprimento e largura do corpo, comprimento do flagelo livre, tamanho e posição do núcleo e do cinetoplasto etc. A posição do cinetoplasto em relação ao núcleo e a presença ou não de membrana ondulante e flagelo livre determinam as diferentes formas apresentadas por estes flagelados durante seus ciclos de vida (Figura 1): amastigota, coanomastigota, promastigota, opistomastigota, epimastigota, tripomastigota e opistomastigota. Formas consideradas estágios de transição ou raramente encontradas, como paramastigotas e endomastigotas, também foram descritas (Wallace et al., 1983; Wallace, 1966).

Foi com base nos parâmetros taxonômicos tradicionais (morfologia, ciclo de vida e hospedeiro de origem) que os flagelados da família Trypanosomatidae foram distribuídos em 10 gêneros: seis que abrigam parasitas monoxênicos de insetos *(Herpetomonas*, *Crithidia*, *Blastocrithidia*, *Leptomonas*, *Wallaceina e Sergeia)*, um que abriga parasitas heteroxênicos de insetos e plantas (gênero *Phytomonas)* e os gêneros heteroxênicos *Leishmania, Endotrypanum* e *Trypanosoma* que abrigam espécies de importância médica e veterinária e que parasitam insetos e vertebrados (Hoare e Wallace; 1966; Merzlyak et al., 2001, Svobodová et al., 2007; Wallace et al., 1983; Wallace, 1966).

Hipóteses filogenéticas baseadas em marcadores moleculares, dados biogeográficos e paleontológicos tentam explicar a origem dos tripanossomatídeos. Foram insetos ou vertebrados seus primeiros hospedeiros? Diferentes estudos sugerem que a adoção do ciclo de vida heteroxênico surgiu independentemente várias vezes ao longo da evolução desses parasitas. Análises filogenéticas recentes de um grande número de espécies dos diferentes grupos de tripanossomatídeos têm

gerado hipóteses filogenéticas bem resolvidas que permitiram sugerir que um bodonídeo ancestral de vida livre pode ter sido ingerido por insetos e se adaptado ao habitat intestinal originando os tripanossomatídeos monoxênicos. Com a aquisição da hematofagia, insetos passaram a inocular esses parasitas em vertebrados e os que se adaptaram ao parasitismo passaram, então, a circular entre insetos hematófagos e vertebrados terrestres, assim originando as espécies heteroxênicas (Hamilton et al., 2004, 2007; Lukeš et al., 2002; Simpson et al., 2006; Stevens et al., 2001; Stevens, 2008).

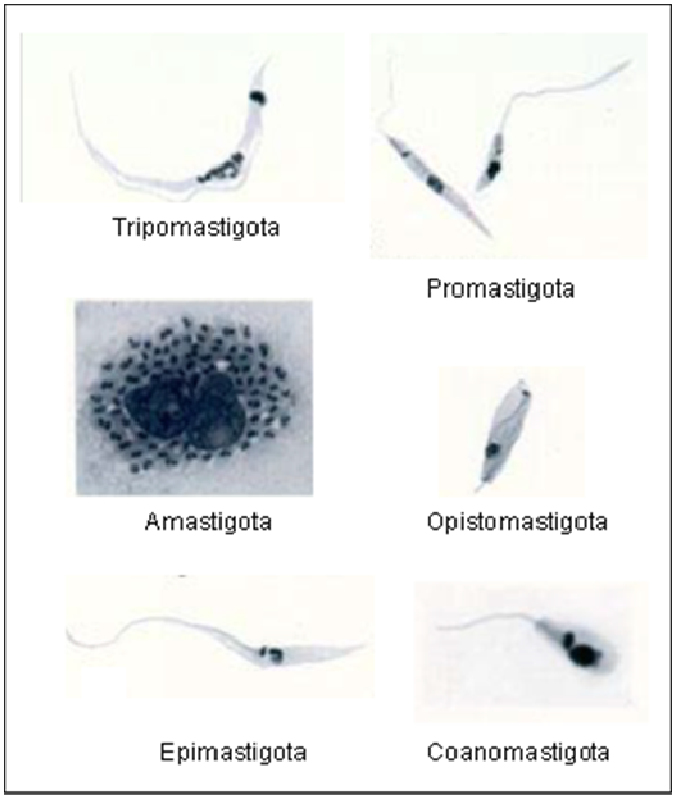


**Figura 1**. Estágios morfológicos encontrados na família Trypanosomatidae (modificada: de Souza W, 2002)

Estudos filogenéticos recentes baseados em diversos marcadores moleculares não têm corroborado grande parte da taxonomia tradicional, questionando muitas das espécies descritas bem como os gêneros da família Trypanosomatidae. Enquanto esses estudos suportam a monofilia dos gêneros *Trypanosoma*, *Leishmania* e *Phytomonas,* os gêneros definidos para os tripanossomatídeos monoxênicos apresentam-se como não monofiléticos. Esses dados mostram que os marcadores taxonômicos tradicionais são insuficientes para

definir a maioria dos gêneros de tripanossomatídeos de insetos (Hollar e Maslov, 1997; Hughes e Piontkivska, 2003; Maslov et al., 2012; Merzlyak et al., 2001; Simpson et al., 2006; Hamilton et al., 2004; 2007; Svobodová et al., 2007; Westenberger et al., 2004; Podlipaev et al., 2004a,b; Yurchenko et al., 2006a,b, 2008; 2009).

## 1.2 Filogenia e taxonomia de tripanossomatídeos parasitas de insetos

Pouco se conhece sobre a diversidade e as relações filogenéticas de tripanossomatídeos monoxênicos. Esses protozoários são os mais abundantes na família e apresentam grande diversidade de hospedeiros, preferencialmente hemípteros e dípteros, tendo sido encontrados em mais de 400 espécies de insetos em todo o mundo. Aproximadamente 80% dos tripanossomatídeos de insetos foram descritos nessas duas ordens (Maslov et al., 2013). Geralmente não são patogênicos, desenvolvem-se no trato digestivo dos insetos (que fornece os nutrientes necessários para a propagação dos parasitas) e são transmitidos por coprofagia, predação e até mesmo canibalismo (Camargo, 1998; Hoare, 1972; Maslov et al., 2013; Vickermam, 1994; Wallace, 1966).

Baseando-se em caracteres morfológicos e ciclo de vida, Hoare e Wallace (1966) reorganizaram os tripanossomatídeos monoxênicos de insetos nos gêneros: *Crithidia, Leptomonas, Herpetomonas* e *Blastocrithidia.* Os gêneros *Wallaceina* originalmente denominado *Proteomonas*, e criado para acomodar tripanossomatídeos de insetos que apresentam formas endomastigotas (morfologia em que o flagelo se enrola dentro da célula, de modo que a extremidade não fique livre) e *Sergeia* foram posteriormente adicionados à família (Merzlyac et al., 2001; Podlipaev et al., 1990; Svobodová et al., 2007, Yoshida et al., 1978). Entre os tripanossomatídeos monoxênicos de insetos, cinco espécies apresentam endossimbiontes bacterianos no citoplasma : *Blastocrithidia culicis* (Chang, 1975), *Crithidia oncopelti* (Gill e Vogel, 1963), *Crithidia deanei* (Carvalho, 1973), *Crithidia desouzai* e *Herpetomonas roitmani* (Fiorini et al., 1989; Faria-e-Silva et al., 1991; Motta et al., 1991). A presença de endossimbiontes bacterianos na família Trypanosomatidae foi também relatada em tripanossomas de peixe, *Trypanosoma cobitis* (Lewis e Ball, 1981), e de anuro, *Trypanosoma fallisi* (Martin e Desser, 1990).

De acordo com parâmetros taxonômicos tradicionais, o gênero *Crithidia* é constituído por tripanossomatídeos que apresentam formas coanomastigotas em seu ciclo de vida. Entretanto, análises filogenéticas baseadas em marcadores moleculares posicionam as espécies desse taxon em dois clados distintos e distantes (Figura 2) indicando que o gênero não é monofilético. Tripanossomatídeos com formas coanomastigotas que albergam endossimbiontes bacterianos (*C. deanei, C. desouzai* e *C. oncopelti*) são segregados juntamente com *Blastocrithidia culicis* e *Herpetomonas roitmani,* que também contêm endossimbiontes bacterianos, em um grupo monofilético muito distante de *C. fasciculata*, espécie-tipo do gênero e que não contém endossimbionte. *Crithidia fasciculata* é proximamente relacionada com espécies dos gêneros *Leishmania*, *Endotrypanum*, *Leptomonas* e *Wallaceina* (Hamilton et al., 2004; Hollar e Maslov, 1997; Hollar et al., 1998; Jirků et al., 2012; Hughes e Piontkivska, 2003; Maslov et al., 2010; 2013; Merzlyak et al., 2001; Svobodová et al. 2007; Yurchencko et al., 2008, 2009). Estes dados indicam que o gênero *Crithidia* é um taxon artificial que necessita ser redefinido e separado em, pelo menos, dois gêneros (Hollar et al., 1998).

*Leptomonas* é outro gênero que necessita de revisão. Análises filogenéticas inferidas com os genes gGAPDH e SSU rRNA têm sugerido a polifilia do gênero e revelado que as espécies *Leptomonas costarricensis* e *Leptomonas barvae* são mais proximamente relacionadas com *Leishmania* do que com o clado que contém *Leptomonas seymouri,* espécie-tipo do gênero (Maslov et al., 2010, Votýpka et al., 2012a; Yurchenko et al., 2006b). Na realidade, o gênero *Leptomonas* juntamente com os gêneros *Crithidia*, *Leishmania*, *Endotrypanum e Wallaceina* formam um grande clado que, devido às taxas relativamente lentas de evolução das sequências de SSU rRNA, foi denominado *Slowly Evolving* (SE) (Merzlyak et al., 2001). Recentemente, com base em filogenias geradas com os genes SSU rRNA e gGAPDH esse táxon foi denominado como Subfamilia Leishmaniinae (Jirků et al., 2012), tendo *Leishmania* (Ross, 1903) como gênero tipo.

Muitas espécies de *Herpetomonas* foram descritas com base em parâmetros taxonômicos tradicionais (Podlipaev 1990; Wallace, 1966; Wallace et al.1983). Contudo, análises filogenéticas demonstraram que as espécies classificadas nesse genêro posicionam-se em ramos distantes e não relacionados (Figura 2). Um exemplo é *H. roitmani* que se posiciona juntamente com os tripanossomatídeos que

têm simbiontes e distantemente do clado das demais espécies desse gênero (*H. mariadeanei*, *H. muscarum*, *H. magaseliae*, *H. samuelpessoai*) (Figura 2). Atualmente, o gênero *Herpetomonas* (comentado separadamente adiante) também é considerado polifilético e requer uma revisão taxonômica.

O gênero *Blastocrithidia* (Laird, 1959) foi criado para acomodar tripanossomatídeos de insetos com formas epimastigotas (Wallace, 1966). Tem como espécie tipo *Blastocrithidia gerridis*, que não foi cultivada e, portanto não está disponível para comparações morfológicas e moleculares. As espécies de *Blastocrithidia* compartilham apenas as formas epimastigotas com as espécies do gênero *Trypanosoma*. Embora espécies de ambos os gêneros infectem triatomíneos, as espécies de *Blastocrithidia* diferem das de *T. cruzi* e *T. rangeli*, por também se desenvolverem em outros insetos. Construções filogenéticas recentes mostram que os flagelados classificados como *Blastocrithidia* com base em parâmetros morfológicos não formam um grupo monofilético. A espécie *B. culicis,* portadora de endossimbionte, agrupa-se no mesmo clado em que se posicionam as outras espécies de tripanossomatídeos portadoras de endossimbiontes. Por outro lado, o clado contendo *B. triatomae*, *B. leptocoridis*, *B. cyrtomeni* e *B. largi* é segregado em um ramo distante e basal dos tripanossomatídeos (Hamilton et al., 2004, Maslov et al., 2012; Merzlyak et al., 2001; Votýpka et al., 2012b, Yurchenko et al., 2008). O status taxonômico do gênero *Blastocrithidia* na família Trypanosomatidae ainda é incerto.

A classificação de tripanossomatídeos monoxênicos de insetos em gêneros revela uma série de dificuldades, sendo realizada, na maioria das vezes, com base em parâmetros taxonômicos tradicionais (morfologia, ciclo de vida e hospedeiros de origem). Além disso, os tripanossomatídeos de insetos apresentam baixa especificidade em relação ao hospedeiro e uma grande diversidade morfológica. Embora parâmetros morfológicos sejam utilizados na definição de gêneros da família Trypanossomatidae, sua utilização para a classificação de tripanossomatídeos monoxênicos de insetos deve ser feita com cautela.

Espécies de mais de um gênero compartilham as mesmas características morfológicas (Tabela 1) e um mesmo gênero pode apresentar diferentes estágios e grandes polimorfismos (Camargo et al., 1992; Camargo, 1999; Faria-e-Silva et al., 1991; Teixeira et al., 1997; Wallace et al., 1983). A existência de infecções mistas

nos insetos e consequentemente nas culturas obtidas é um dos principais fatores que complicam a identificação morfológica de tripanossomatídeos. Formas observadas diretamente nos hospedeiros nem sempre correspondem às isoladas em cultura, que podem ser de outras espécies (minoritárias quando no hospedeiro de origem, porém facilmente cultiváveis), ou até mesmo de outros gêneros. Além da morfologia, a espécie do hospedeiro de origem é um critério amplamente utilizado na classificação de tripanossomatídeos. Entretanto, já foi demonstrado experimentalmente que tripanossomatídeos de insetos apresentam diferentes graus de especificidade em relação aos hospedeiros. Além disso, um hospedeiro pode conter infecções múltiplas, ou seja, albergar diferentes espécies de tripanossomatídeos (Camargo, 1999; Podlipaev, 2001; McGhee e Hanson, 1963; McGhee, 1970; Podlipaev et al., 2004a; Týč et al., 2013; Votýpka et al., 2012a; Wallace et al., 1983; 1993; Wallace, 1966; Zídková et al., 2010). Recentemente Votýpka e colaboradores (2012b) demonstraram que *Leptomonas pyrrhocoris* tem distribuição cosmopolita e apresenta especificidade com hospedeiros pertencentes à família Pyrrhocoridae. Essa família de hemíptero possue gêneros de distribuição mundial, o que possibilitou a disseminação do flagelado. Esses dados demonstram a importância de estudos filogeográficos para os tripanossomatídeos de insetos.

**Tabela 1**- Tripanossomatídeos monoxênicos de insetos

| Gênero | Morfologia | Hospedeiro |
|---|---|---|
| *Crithidia* | Coanomastigota | Hemiptera/Diptera |
| *Leptomonas* | Promastigota | Hemiptera |
| *Herpetomonas* | Promastigota<br>Opistomastigota | Hemiptera/Diptera |
| *Blastocrithidia* | Epimastigota | Hemiptera |
| *Sergeia* | Promastigota | Diptera |
| *Wallaceina* | Promastigota<br>Endomastigota | Hemiptera |

Marcadores moleculares têm sido amplamente utilizados para a análise da diversidade dos tripanossomatídeos, mostrando-se muito mais confiáveis que os marcadores tradicionais tanto para a identificação de espécies ou cepas de

organismos conhecidos quanto para a classificação de novas espécies ou a criação de táxons supraespecíficos. O posicionamento em diagramas filogenéticos tem permitido a identificação de novas espécies morfologicamente indistinguíveis de outras já conhecidas. Por outro lado, espécies originalmente classificadas como distintas tem sido sinonimizadas com base em dados moleculares (Ferreira et al. 2007, 2008; Hamilton et al., 2004, 2005a,b, 2007, 2009; Lima et al., 2012; Maia da Silva et al. 2004a,b; Maslov et al., 2012; Podlipaev et al., 2004b; Rodrigues et al. 2006, 2008; Stevens et al. 1999a,b, 2001; Svobodová et al., 2007; Viola et al., 2008, 2009a,b; Zídková et al., 2010).

Nos estudos evolutivos da família Trypanosomatidae, embora um número crescente de espécies venha sendo incluída ao longo do tempo, principalmente as do gênero *Trypanosoma,* ainda são poucos os representantes dos diferentes gêneros de tripanossomatídeos monoxênicos de insetos. Nos últimos anos aumentaram consideravelmente os estudos dedicados a explorar comparativamente a diversidade destes tripanossomatídeos de insetos (Maslov et al., 2007; Podlipaev et al., 2004a,b; Svobodová et al., 2007; Votýpka et al., 2010; Yurchenko et al., 2006a,b, 2008, 2009). Esses estudos apontam a necessidade de uma completa revisão taxonômica de praticamente todos os gêneros de tripanossomatídeos monoxênicos. Para isso, um grande número de isolados representativos da grande diversidade de hospedeiros e regiões geográficas precisam ser analisados.

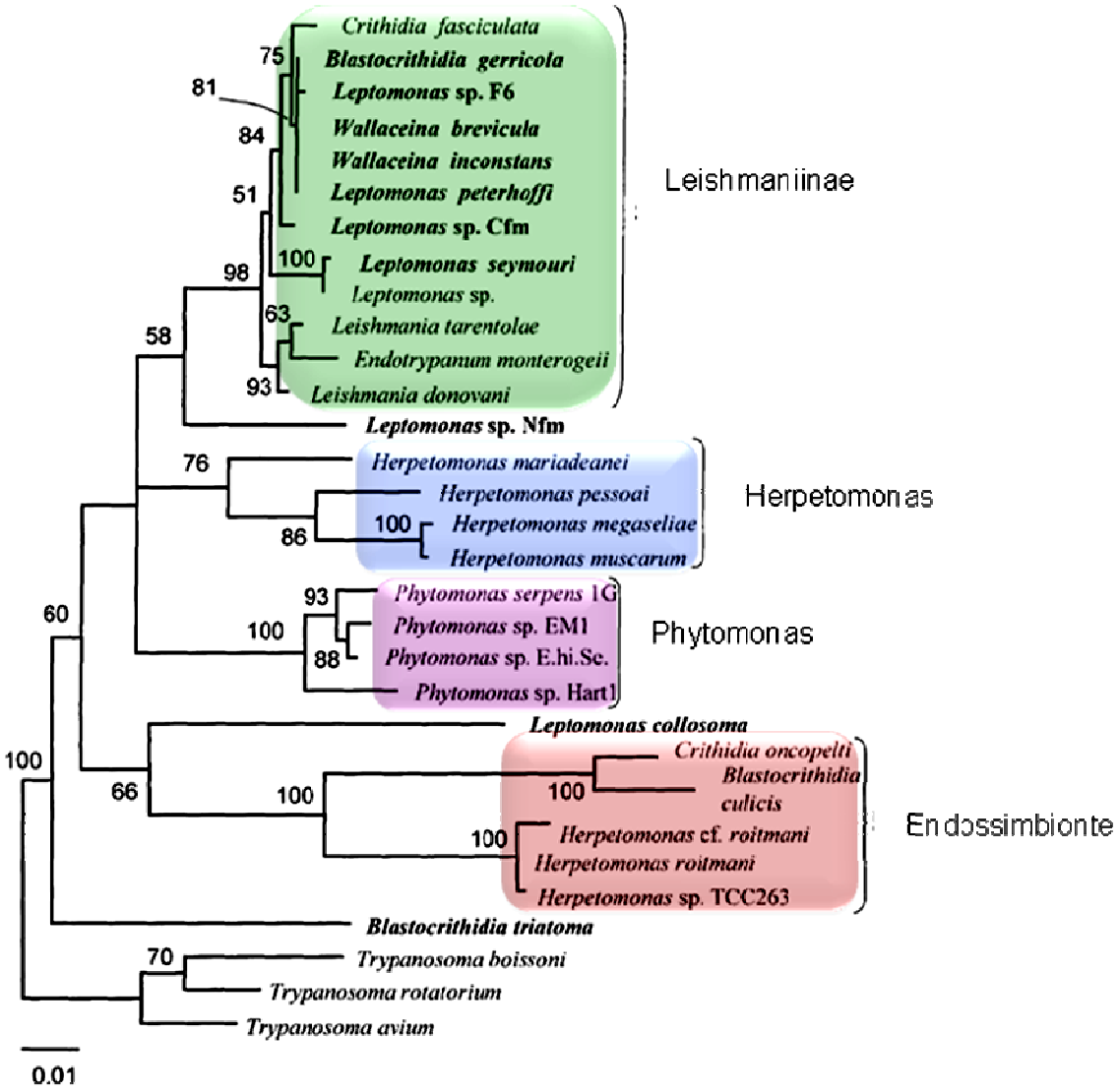


**Figura 2**. Diagrama de máxima verossimilhança (ML) baseada no gene SSU rRNA utilizando sequências de tripanossomatídeos. Os números correspondem aos valores de suporte por "bootstrap" em ML inferidos por 100 replicatas. Fonte: modificada de Merzlyak et al., 2001.

### 1.3 Tripanossomatídeos parasitas de moscas

A classe Insecta é formada por mais de 750.000 espécies distribuídas em pelos menos 10 ordens. Cerca de 200 espécies foram descritas com infecções por tripanossomatídeos, em mais de 300 relatos em todo o mundo (Wallace et al., 1983; Sbravate et al., 1989; Teixeira et al., 1997; Serrano et al., 1999; Camargo et al., 1992; Camargo, 1998; Podlipaev et al., 2004a,b; Maslov et al., 2007, Votýpka et al., 2012).

A ordem Diptera, uma das maiores ordens da classe Insecta, constitui um grupo monofilético que abriga cerca de 158.000 espécies que apresentam grande

variação morfológica e diversidade ecológica. Os dípteros conhecidos atualmente estão classificados em pelo menos 130 famílias, 22-32 superfamílias, 8-10 infraordens e 5 subordens (McAlpine e Wood, 1989; Colless e McAlpine, 1991; Yaetes e Wiegmann, 1999, Amorim e Yaetes, 2006; Wiegmann et al., 2003). Somente uma pequena parte da diversidade dos dípteros foi examinada quanto à presença de tripanossomatídeos. Portanto, os estudos sobre a diversidade de tripanossomatídeos de moscas ainda são muito restritos, pois representam apenas uma pequena fração da diversidade de hospedeiros.

Os fósseis mais antigos de dípteros datam do Permiano superior (~250 mya) e um grande número de fósseis de Diptera datam do Mesozóico (Yeates e Wiegmann, 1999; Wiegmann et al., 2011). Os dipteros são considerados uma das maiores radiações adaptativas entre os eucariotos terrestres. Análises filogenéticas recentes baseadas em dados moleculares, morfológicos e fósseis sugerem que a grande diversidade de dípteros (tanto morfológica quanto ecológica) é devida a episódios de rápidas explosões radiativas, ou seja, os dipteros evoluíram e se diversificaram dos seus ancestrais em um curto período de tempo (Wiegman et al., 2011)

A maioria dos dípteros adultos alimenta-se de pólen e néctar de plantas ou de matéria orgânica em decomposição. Devido aos diferentes hábitos dos estágios larvais (detritívoros, semiaquáticos ou aquáticos), algumas espécies de moscas podem causar danos econômicos, tanto para plantas (caso da mosca das frutas, da família Tephritidae, cujas larvas danificam principalmente os frutos) quanto para animais (caso de moscas das famílias Calliphoridae e Oestridae, cujas larvas podem invadir tecidos vivos de vertebrados, causando miíases) (Yates e Wiegmann, 1999, Stevens et al., 2006). Insetos adultos das famílias Simuliidae, Culicidae, Psychodidae e Glossinidae têm hábitos hematófagos, constituindo-se em importantes hospedeiros e/ou vetores de oncocercose, malária, leishmaniose e tripanossomíases africanas (doença do sono), respectivamente (Yates e Wiegmann, 1999, Rodrigues et al., 2006). Por outro lado, algumas espécies de dípteros (Tephritidae, Tachinidae) possuem larvas com hábitos parasitóides (parasitam outras espécies de insetos) e desempenham importante papel na regulação de pragas, sendo utilizadas no controle biológico de insetos e plantas (Yates e Wiegmann, 1999).

Tradicionalmente, os dípteros estão divididos nas subordens Nematocera e Brachycera. A subordem Nematocera (com seis infraordens), da qual fazem parte os mosquitos (Culicidae), borrachudos (Simuliidae) e flebotomíneos (Psycodidae), contém os dípteros com antenas com mais de seis segmentos livremente articulados. Análises filogenéticas baseadas em dados moleculares e morfológicos sugeriram que essa subordem é parafilética e que o relacionamento entre as infraordens é incerto (Yates e Wiegmann et al., 1999, Wiegmann et al., 2003). A subordem Brachycera constitui um grupo monofilético, formado por dípteros com antenas com 3 a 5 segmentos e distribuídos em quatro infraordens: Xylophagomorpha, Stratiomyomorpha, Tabanomorpha e Muscomorpha (antiga subordem Cyclorrapha), que compreende os dípteros muscóides. Com base em dados morfológicos, a Infraordem Muscomorpha divide-se em quatro grandes grupos: Heterodactyla, Eremoneura (formado por Empidoidea e Cyclorrapha), Cyclorrapha e Schizophora (Figura 3) (Yates e Wiemann et al., 1999). Eremoneura forma o clado mais bem suportado dentro da subordem Brachycera (Yates e Wiegmann 1999; Wiegmann et al., 2011).

De acordo com a análise de caracteres morfológicos, Cyclorrapha é dividido em duas seções: Aschiza e Schyzophora. A seção Aschiza é formada por duas superfamílias: Syrphoidea (formada por duas famílias) e Platypezoidea (composta por cinco famílias). Estudos recentes baseados em dados moleculares têm demonstrado que essa seção é parafilética (Yates e Wiegmann 1999; Wiegmann et al., 2011). Na seção Schizophora, encontra-se a maior diversidade de famílias da ordem Díptera, com aproximadamente 85 famílias divididas em duas subseções: Acalyptratae e Calyptratae. Com aproximadamente 18.000 espécies descritas, a seção Calyptratae consiste em um dos maiores e mais diversificado grupo da ordem Diptera, com todos os representantes posicionados em um único clado fortemente suportado (Yates e Wiegmann 1999; Wiegmann et al., 2011). McAlpine (1989) dividiu a seção Calyptratae em três superfamilias: a) Hippoboscoidea, composta pelas famílias Glossinidae, Hippoboscidae, Strablidae e Nycteribiidae; b) Muscoidea, que inclui Sconthophagidae, Anthomyiidae, Faniidae e Muscidae; e c) Oestroidea, formada pelas famílias Calliphoridae, Mystacinobiidae, Sarcophagidae, Rhinophoridae, Tachinidae e Oestridae.

Moscas de diversas espécies, gêneros e famílias são hospedeiras e/ou vetores de tripanossomatídeos heteroxênicos. Diversos fósseis de Diptera, de diferentes períodos, foram encontrados infectados por tripanossomatídeos (Poinar e Poinar, 2005). O ciclo biológico dos tripanossomatídeos nos insetos ocorre em diferentes porções do tubo digestivo e, em alguns casos, nas glândulas salivares. As espécies africanas do gênero *Trypanosoma* pertencentes à secção Salivaria são parasitas de mamíferos que têm moscas tsé-tsé (glossinas) como vetores. No caso de tripanossomas do clado *T. theileri,* parasitas de Artiodactyla, os vetores da transmissão contaminativa são moscas das famílias Tabanidae e Hippoboscidae (Rodrigues et al., 2006). Pequenas moscas hematófagas pertencentes à família Ceratopogonidae transmitem algumas espécies de tripanossomas de aves (Votýpka et al., 2002), mas também foram descritas infectadas por tripanossomatídeos monoxênicos dos gêneros *Herpetomonas* e *Sergeia* (Podlipaev et al., 2004b*;* Svobodová et al., 2007; Zídková et al., 2010). Estudos recentes têm demonstrado que tripanossomatideos são comuns em diversas espécies de *Drosophila* (Ebbert et al., 2001; Wilfert et al, 2011). Em um estudo prévio de nosso laboratório, com base em polimorfismos de restrição do gene SSU rRNA, classificamos 11 tripanossomatídeos isolados de moscas (pertencentes às famílias Muscidae e Calliphoridae) no gênero *Herpetomonas* (Teixeira et al., 1997).

Centenas de tripanossomatídeos monoxênicos foram descritos parasitando moscas de diversas espécies e famílias. Entretanto, poucos estudos foram realizados a fim de classificar esses flagelados e, na maioria dos casos, a classificação foi baseada apenas em dados morfológicos (Faria e Silva et al., 1991;Hollar et al., 1998; Podlipaev et al., 2004a,b; Yurchenko et al., 2006a,b, 2008). Os flagelados estudados neste trabalho foram isolados de moscas principalmente da família Calliphoridae, mas também das famílias Muscidae, Sarcophagidae e Syrphidae (figura 3).

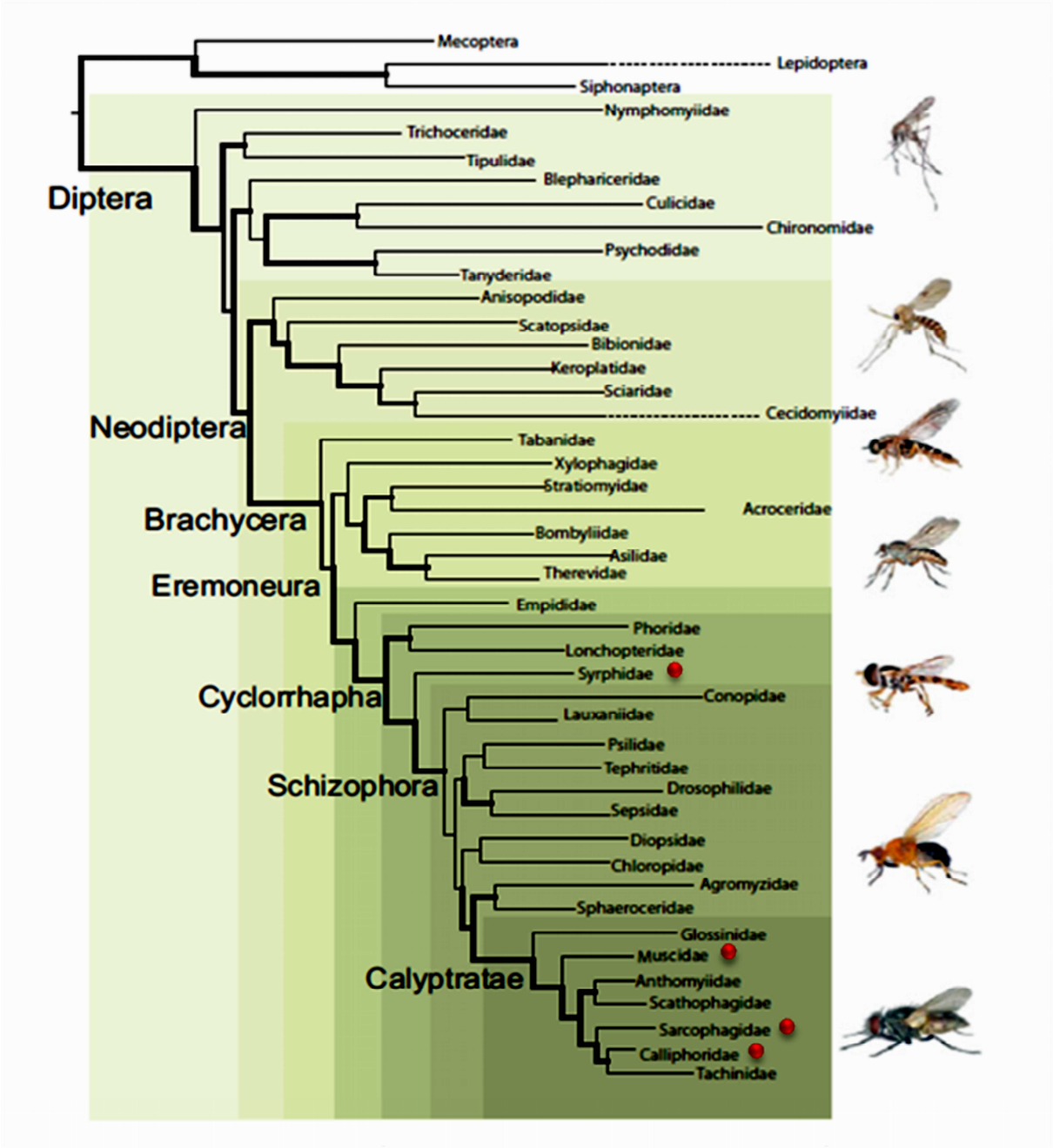


**Figura 3**: Inferência filogética de Diptera baseada em dados morfológicos e moleculares. Bolas vermelhas indicam as famílias onde os isolados deste estudo foram encontrados (modificada de Wiegmann et al, 2003)

A família Calliphoridae, que compreende moscas com reflexos metálicos (geralmente azulados e esverdeados) conhecidas como “moscas varejeiras” ou “blowfly” (Guimarães e Papavero, 1999), forma um grupo muito diverso e heterogêneo, havendo, aproximadamente, 1.500 espécies distribuídas mundialmente (Marinho et al., 2012; Pape et al., 2011). As espécies de Calliphoridae das regiões neotropicais são agrupadas em quatro subfamílias: Chrysomynae,

Calliphorinae, Mesembrineliinae e Toxotarsinae, com ~27 gêneros e mais de 125 espécies. As principais espécies causadoras de miíases são encontradas nos gêneros *Cochliomyia, Lucilia, Calliphora, Compsomyiosps* e *Chrysomya* (de Azeredo-Espin e Lessinger, 2006; Guimarães e Papavero, 1999; Stevens e Wall, 1996; Stevens et al., 2006a,b). As espécies mais comuns no Brasil, com grandes populações sinantrópicas, são espécies exóticas dos gêneros *Luciliae* (Calliphorinae) e *Chrysomya* (Chrysomynae). Mesembrineliinae e Toxotarsinae são subfamilias neotropicais restritas às regiões silvestres e são muito utilizadas como marcadores de impacto ambiental (Guimarães e Papavero, 1999; Melo, 2003).

As larvas de Calliphoridae desenvolvem-se em locais diversos: em invertebrados, materiais em decomposição (fezes, lixo, carcaças de animais, etc) e tecidos vivos de vertebrados. Algumas espécies são atraídas também por flores e frutas com aromas acentuados. Devido ao seu papel como ectoparasitas de animais (causando miíases primárias e secundárias) e sua importância na entomologia forense, espécies de Calliphoridae tem sido bastante estudadas filogeneticamente (Stevens, et al., 2006a,b; Stevens 2003; de Azeredo-Espin e Lessinger, 2006; Marinho et al., 2012).

Enquanto algumas espécies de Calliphoridae são endêmicas, outras foram introduzidas pelo homem e estão dispersas atualmente em praticamente todos os continentes. No Brasil, as espécies endêmicas são principalmente de hábitos silvestres e existem em pequenas populações. O gênero *Cochliomyia* é endêmico no novo mundo e inclui importantes agentes causadores de miíases obrigatórias. As espécies *Cochliomyia hominivorax* e *Cochliomyia macellaria* são as mais abundantes e amplamente distribuídas e atualmente são encontradas em populações pequenas e restritas a regiões rurais (Stevens et al., 2003; 2006a,b; de Azeredo-Espin e Lessinger, 2006). O gênero *Chrysomya* era restrito ao Velho Mundo até meados da década de 1970, quando foram descritas três espécies no Brasil: *C. megacephala* (originária da Austrália e Oriente), *C. albiceps* (originária da Europa) e *C. putoria* (originária da África). Essas espécies possuem uma grande capacidade de adaptação a novos ambientes, o que levou a uma rápida dispersão e a ocorrência de populações sinantrópicas. Atualmente, essas são as espécies mais abundantes no Brasil. Com o estabelecimento dessas espécies no Brasil, ocorreu o deslocamento de espécies endêmicas para ambientes silvestres (Guimarães, 1999;

de Azeredo-Espin e Lessinger, 2006). Estudos recentes, baseados em dados morfológicos e moleculares, demonstram que a familia Calliphoridae é parafilética (Kutty et al., 2008; Marinho et al., 2012).

A família Muscidae é composta por aproximadamente 5.000 espécies, distribuídas em cerca de 170 gêneros. As espécies dessa família são encontradas em todas as regiões biogeográficas. Suas larvas possuem hábitos saprófagos e quando adultos podem ser saprófagos, predadores, hematófagos ou alimentar-se de néctar e pólen. Estudos recentes baseados em dados moleculares demonstram que Muscidae é monofilética, dividida em oito subfamílias: Achanthipterinae, Atherigoninae, Azeliinae, Cyrtoneurininae, Coenosiinae, Muscinae, Mydaeinae e Phaoniinae (Kutty et al., 2008). A espécie mais conhecida da família é *Musca domestica.* Essa espécie é cosmopolita, sinantrópica e têm um importante papel como vetor de microorganismos (Gupta et al., 2012).

### 1.4 Tripanossomatídeos que albergam simbiontes

A maioria dos tripanossomatídeos que albergam simbiontes pertence ao gênero *Crithidia*, gênero criado no início do século XX para classificar tripanossomatídeos com formas de “grão de cevada”, ligeiramente achatados na extremidade anterior de onde se exterioriza o flagelo. *Crithidia fasciculata* é a espécie tipo (Léger, 1902). Durante muito tempo o termo “critidial stage” foi adotado para descrever em outros gêneros formas similares às encontradas no gênero *Crithidia*, gerando grande confusão e inúmeros erros de classificação. Apesar de a morfologia coanomastigota ser considerada exclusiva do gênero *Crithidia* a identificação de coanomastigotas nem sempre é fácil, quando se considera o grande pleomorfismo dos indivíduos desse gênero (Wallace, 1966; Wallace et al., 1983) (Figura 4). Exceto em *B. culicis* (que apresenta forma epimastigota), nos demais tripanossomatídeos que albergam endossimbiontes bacterianos (*C. deanei*, *C. desouzai*, *C. oncopelti* e *H. roitmani*) encontram-se formas nas quais o cinetoplasto localiza-se posteriormente ao núcleo, presentes em maior número em culturas em fase estacionária (Teixeira et al., 1997) (Figura 4). Estas formas, que aqui designamos opistomorfas, não são encontradas nos tripanossomatídeos sem endossimbiontes do gênero *Crithida*. O termo opistomorfa foi adotado para distinguir

as formas coanomastigotas com cinetoplasto posterior ao núcleo das formas opistomastigotas típicas, que são formas alongadas, como as promastigotas, e exclusivas das espécies do gênero *Herpetomonas* (Teixeira et al., 1997).

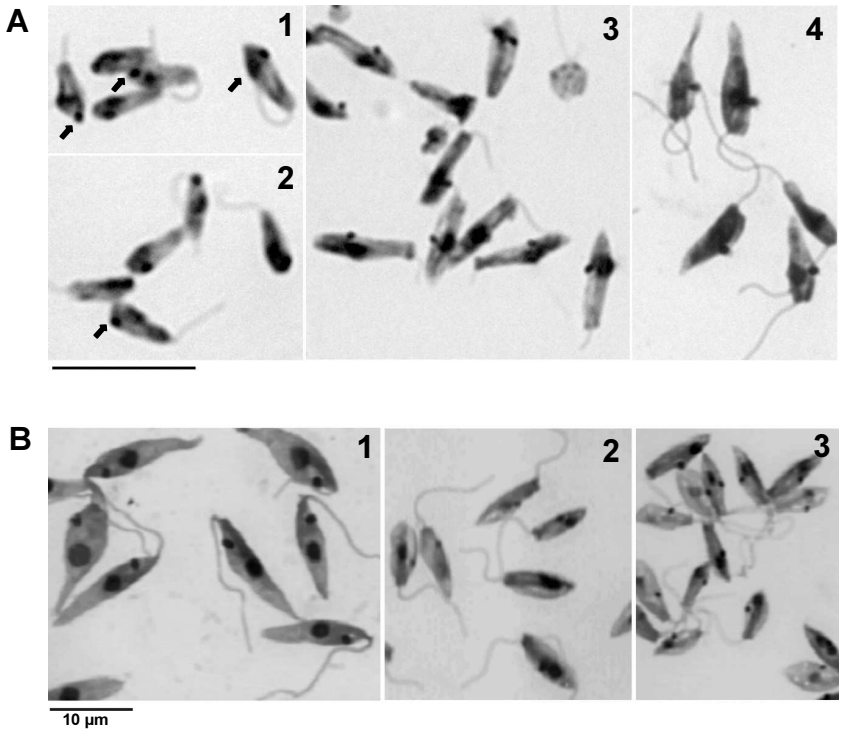


**Figura 4**: Microscopia de luz de tripanossomatídeos do gênero *Crithidia* portadores de simbiontes: **A**: 1 e 2 *C. deanei*; 3) *C. desouzai*; 4) *C. oncopelti;* e não portadores de simbiontes **B**: 1) *C. fasciculata*; 2) *C. acanthocephali* e 3) *C. l. thermofila*. As setas indicam formas opistomorfas.

Os endossimbiontes de tripanossomatideos pertencem à classe Betaproteobacteria, são intracitoplasmáticos obrigatórios, não sobevivem fora do hospedeiro e são circundados por uma dupla membrana separada por um espaço eletronlúcido (Chang, 1974; 1975; Du et al., 1994a, 1994b; Hollar et al., 1998; Tuan e Chang, 1975; Alfieri e Camargo, 1982; De Souza e Motta, 1999). Geralmente há apenas um endossimbionte por célula o qual se divide sincronicamente com seu hospedeiro (Du et al., 1994b; Motta et al., 2010). Estudos sobre requerimentos nutricionais demonstraram a contribuição dos simbiontes para o metabolismo dos tripanossomatídeos hospedeiros fornecendo vários nutrientes essenciais (Chang e Trager, 1974; Frossard et al., 2006; Korený et al., 2010; Roitman e Camargo, 1985). Tripanossomatídeos que contêm endossimbiontes bacterianos não exigem adição de ornitina, arginina e citrulina no meio de cultura, intermediários do ciclo da uréia que são supridos pelo endossimbionte (Galinari e Camargo, 1978; Camargo e

Freymuller, 1977). Os endossimbiontes também influenciam na síntese de fosfatidilcolina (de Azevedo-Martins et al., 2007), ajudam na captação de prolina (Galvez Rojas et al., 2008) e sintetizam treonina-desaminase, primeira enzima da via biossintética de isoleucina, leucina e valina, envolvida na conversão de treonina em alfa-cetobutirato. Assim, espécies com endossimbiontes sobrevivem na ausência desses aminoácidos, o que não ocorre com *C. fasciculata,* cujo desenvolvimento é bastante prejudicado (Alfieri e Camargo, 1982).

Enquanto a imensa maioria dos tripanossomatídeos requer hemina como fonte de heme (uma molécula encontrada em todos os seres vivos e de fundamental importância funcional), as espécies portadoras de simbionte dispensam hemina no meio de cultura, indicando a capacidade do simbionte de sintetizar o heme. A via de síntese de heme está ausente na maioria dos cinetoplastidas, enquanto enzimas e precursores envolvidos no percurso biossintético são mais abundantes em tripanossomatídeos com endossimbiontes (Newton, 1957; Chang e Trager, 1974; Mundim e Roitman, 1977; Mundim et al., 1974; Faria-e-Silva et al., 1994). Análises genômicas conduzidas recentemente demostraram que os endossimbiontes de tripanossomatídeos possuem a via de biossíntese de heme potencialmente completa (Alves et al., 2011), o que explicaria a propagação em cultura dos tripanossomatídeos hospedeiros em meios desprovidos de hemina (Mundim e Roitman, 1977; Mundim et al., 1974; Alfieri e Camargo, 1982; Faria-e-Silva et al., 1994). A via completa da síntese desse composto é encontrada na maioria dos organismos e é altamente conservada (Korený et al., 2010; 2013). Embora raros, alguns organismos apresentam deficiências na síntese deste composto, como é o caso de protistas anaeróbicos como *Giardia*, *Entamoeba*, *Trichomonas,* entre outros (Korený et al., 2010; 2013). Por outro lado, estudos recentes baseados em análises de genomas completos mostraram a presença, em espécies de *Leishmania* e *Crithidia*, dos genes que codificam as três últimas enzimas da via biossintética do heme (Alves et al., 2011.), provavelmente oriundos de uma transferência horizontal de Gammaproteobacteria. Filogenias baseadas nos genes da via biossintética do heme sugerem que a via heme tenha sido perdida no ancestral ainda não parasita dos cinetoplastidas e mais tardiamente readquirida, por transferência horizontal, em *Leishmania* e *Crithidia*. Nos tripanossomatídeos que albergam simbiontes, a

aquisição dos genes oriundos de gammaproteobacteria ocorreu antes do evento de simbiose com betaproteobacteria.

Os flagelados que albergam endossimbiontes diferem dos que não possuem endossimbiontes em muitas características morfológicas, bioquímicas e moleculares (Brandão et al., 2000; Camargo et al., 1982, 1992; Fernandes et al., 1997; Hollar et al., 1998, Maslov et al., 2010; Merzlyak et al., 2001; Teixeira et al., 1995). Além de conferir vantagens nutricionais e metabólicas, o endossimbionte altera a morfologia e ultra-estrutura dos tripanossomatídeos hospedeiros (de Souza e Motta 1999; Motta et al., 1997) de forma que os tripanossomatídeos portadores de simbiontes diferem dos não portadores em muitas características ultraestruturais (Cavalcanti et al., 2008; Freymuller e Camargo, 1981), por exemplo: ausência de estrutura paraflagelar; alterações na organização do cinetoplasto com afrouxamento da rede de kDNA; e ausência de microtúbulos subpeliculares nas regiões em que ramificações da mitocôndria se aproximam da porção interna da membrana plasmática (Figura 5).

Quando tratados com cloranfenicol, os tripanossomatídeos ficam livres de seus endossimbiontes (cepas apossimbióticas). Entretanto, perdem muitas das suas capacidades biossintéticas (Roitman e Camargo, 1985) e também apresentam diferenças nos glicoconjugados de superfície e enzimas proteolíticas (d'Avila-Levy et al., 2001, 2008). Entretanto, as alterações ultraestruturais acima citadas também são encontradas nas cepas apossimbióticas (Freymuller e Camargo, 1981; Roitman e Camargo, 1985; de Souza e Motta, 1999).

O status taxonômico dos tripanossomatídeos com endossimbiontes tem sido controverso e algumas propostas têm sido sugeridas. Baseando-se nas formas coanomastigotas que apresentam o cinetoplasto localizado posteriormente ao núcleo, Souza e Côrte-Real (1991) propuseram a reclassificação de *C. deanei* em um novo gênero chamado *Angomonas*. Posteriormente com base em características morfológicas e perfis de kDNA, Brandão e colaboradores (2000) propuseram a adoção do gênero *Strigomonas* para acomodar *C. oncopelti* (espécie inicialmente classificada como *Leptomonas* subgênero/gênero *Strigomonas* por Lowff e Lowff (1963) e *Angomonas* para *C. deanei* e *C desouzai*. Entretanto devido à falta de dados taxonômicos suportados por análises filogenéticas moleculares, essa classificação não foi adotada.

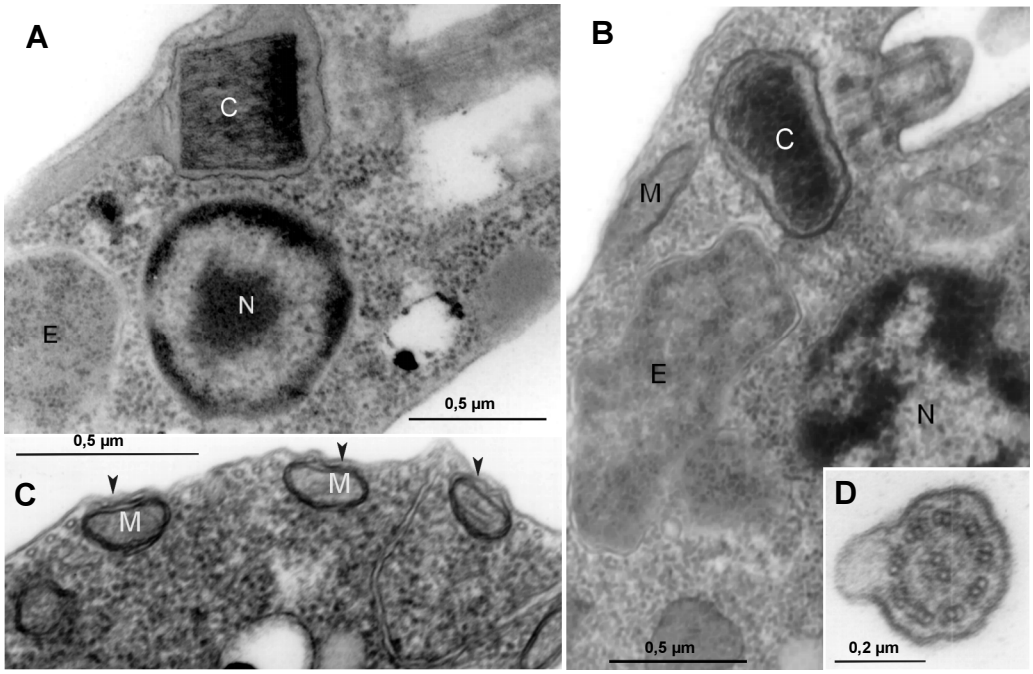


**Figura 5**. Microscopia eletrônica de transmissão. Características ultraestruturais de tripanossomatídeos portadores de endossimbionte bacteriano. Rede de kDNA frouxa (A, B); ausência de microtúbulos subpeliculares nas regiões em que ramificações da mitocôndria se aproximam da porção interna da membrana plasmática indicada pelas setas (C); estrutura paraflagelar ausente (D). Note diferenças na compactação do cinetoplasto em *C. deanei* (A) e *C. oncopelti* (D). Endossimbionte (E), mitocôndria (M), núcleo (N).

### 1.4.1 Filogenia dos endossimbiontes de tripanossomatídeos

A endossimbiose bacteriana em tripanossomatídeos foi inicialmente descrita em *Blastocrithidia culicis* e seus simbiontes foram chamados de diplossomos (Novey et al., 1907). Posteriormente Newton e Horne (1957) reportaram estruturas reprodutivas no citoplasma de *C. oncopelti,* as quais foram denominadas de corpos bipolares. Nos endossimbiontes de *C. oncopelti* foi comprovada pela primeira vez a presença de DNA similar ao de bactérias. A origem procariótica destas estruturas foi confirmada pelo tratamento com antibiótico, que "curou" o flagelado eliminando os corpos bipolares (Chang, 1974; Roitman e Camargo, 1985; De Souza e Motta, 1999). Desde então, diversos estudos tem sido realizados a fim de entender o relacionamento dos endossimbiontes com os tripanossomatídeos e a origem desta simbiose (Freymuller e Camargo, 1981; Du et al., 1994a,b; Hollar et al., 1998; Motta et al., 1997; 2004; De Souza e Motta, 1999).

A primeira análise filogenética de endossimbiontes de tripanossomatídeos foi realizada por Du e colaboradores (1994a; b). Nesses estudos, foram obtidas

sequências de 16S e ITS ribossômicos dos endossimbiontes de C. *deanei*, *C. desouzai, C. oncopelti* e *B. culicis*. Os resultados demonstraram que os endossimbiontes das três espécies de *Crithidia* eram idênticos e muito similares ao de *B. culicis*, e que as sequências destes simbiontes apresentavam grande similaridade com a sequência de *Bordetella bronchiseptica,* pertencente à divisão betaproteobacteria. A distância genética entre o simbionte de *B. culicis* e os endossimbiontes de *Crithidia* spp. é similar à encontrada entre espécies de *Bordetella.* Desta forma, os endossimbiontes de *Blastocrithidia* e *Crithidia* spp. foram considerados betaproteobacteria e classificados como *Kinetoplastibacterium blastocrithidii* e *Kinetoplastibacterium crithidii*, respectivamente (Du et al., 1994a,b). Entretanto, os genes FtsZ (de origem procarótica e homólogos aos de tubulina dos eucariotos) foram observados nos endossimbiontes de *C. deanei* e *B. culicis*, sendo as sequências muito similares nas duas espécies e relacionadas com *Pseudomonas putida* que é pertencente a divisão gammaproteobacteria (Motta et al., 2004), indicando assim uma origem diferente para os simbiontes.

Estudos recentes, baseados em análises dos genomas completos dos simbiontes de tripanossomatídeos, mostraram a total sintenia dos genes das espécies de endossimbiontes e confirmaram que estes pertencem a divisão betaproteobacteria, família Alcaligenacea (Alves et al., 2013, Motta et al., 2013). Contudo, esses endossimbiontes são mais proximamente relacionados com o gênero *Taylorella* do que com o gênero *Bordetella* (Alves et al., 2013). Esses estudos também demonstraram que os simbiontes mantiveram preferencialmente os genes necessários para a biossíntese de compostos necessários aos hospedeiros e que genes relacionados com a cooperação com o hospedeiro são mais comumente retidos no genoma e apresentam pressão positiva de seleção.

Análises filogenéticas baseadas em genes ribossômicos demonstraram que estes simbiontes (16S rRNA) são tão filogeneticamente relacionados quanto seus hospedeiros (SSU rRNA). Esses dados sugerem que a simbiose desses tripanossomoatídeos se originou em um único evento, com uma história evolutiva antiga (provavelmente em um tempo em que o protozoário ancestral era capaz de realizar atividades como fagocitose) no qual houve a aquisição de uma betaproteobacteria por um hospedeiro ancestral, seguido de co-evolução com seus respectivos hospedeiros (Du et al. 1994a,b).

Além de simbiontes bacterianos, algumas espécies da família Trypanosomatidae podem albergar também partículas virais. Diversos estudos demonstraram a presença de partículas virais denominadas LRV pertencentes à familia Totiviriidae em algumas espécies do gênero *Leishmania* (Tarr et al, 1988; Widmer et al, 1989; Gupta e Deep, 2007). Estudos filogenéticos baseados em sequências de vírus dsRNA mostraram que há dois grupos de vírus em leishmânias: LRV1 encontrado em algumas espécies de *Leishmania* do subgênero *Viannia* (novo mundo) e LRV2 encontrado em *L. major* (velho mundo). As distâncias genéticas entre LRV1 e LRV2 são muito semelhantes às distancias genéticas dos parasitas hospedeiros. Assim, esses dados indicam que os vírus provavelmente estavam presentes nas leishmanias antes da divergência entre o novo e velho mundo, e que os simbiontes co-evoluiram com seus hospedeiros (Scheffter et al, 1995;. Widmer e Dooley 1995; Zamora et al., 2000). Estudos recentes demonstraram que a presença do vírus LRV1 pode ser um dos principais fatores que contribuem para o potencial metastático de leishmanias do subgênero *Viannia* (Ives et al, 2011;. Ronet et al; 2011). Entretanto a relação do vírus LRV2 com o parasita ainda é desconhecida (Hartley et al., 2012). *Crithidia desouzai* é a única espécie de tripanossomatídeo que alberga simultaneamente simbiontes bacterianos e partículas virais. Análises ultraestruturais e de imunohistoquímica mostraram partículas semelhantes a vírus (VLPs) no citoplasma de *C. desouzai*. Essas partículas são formadas por RNA, tem formato hexagonal e geralmente ficam mais próximas do núcleo (Motta et al., 2003).

## 1.5 Gênero Herpetomonas

O gênero *Herpetomonas* foi criado para acomodar tripanossomatídeos parasitas monoxênicos de insetos que apresentam formas promastigotas (cinetoplasto localizado anteriormente ao núcleo) e opistomastigotas (cinetoplasto posterior ao núcleo) em seus ciclos de vida (Hoare e Wallace, 1966). Criar um novo gênero para acomodar flagelados apresentando formas opistomastigotas nos insetos e em culturas deveria facilitar a identificação de *Herpetomonas*. No entanto, formas opistomastigotas são raramente encontradas ou até mesmo ausentes tanto em insetos como em culturas, podendo, além disso, ser confundidas com as formas opistomorfas, estas últimas encontradas nos tripanossomatídeos que albergam

simbiontes (Teixeira et al., 1997). Estas incertezas geraram inúmeras classificações equivocadas de espécies de *Herpetomonas* em gêneros que também apresentam formas promastigotas como *Leptomonas* e *Phytomonas* (Teixeira et al., 1997). A maioria dos flagelados descritos como *Herpetomonas* foram isolados de dípteros (Teixeira et al., 1997;. Wallace, 1966). Entretanto, há relatos de flagelados pertencentes a esse gênero encontrados em hemípteros predadores (Roitman et al., 1976), plantas (Fiorini et al., 2001) e até mesmo em humanos imunodeprimidos (Chicharro e Alvar, 2003; Morio et al., 2008). Em seus hospedeiros mais comuns, os dípteros, formas promastigotas vivem no trato digestivo, preferencialmente no reto, podendo ser encontradas livres ou ligadas às células endoteliais (Hupperich et al., 1992; Nayduch, 2009).

Baseando-se nos critérios taxonômicos tradicionais, centenas de espécies de *Herpetomonas* foram descritas (Podlipaev, 1990; Wallace, 1966; Wallace et al.,1983). Entretanto, inferências filogenéticas recentes revelaram que os flagelados classificados pela taxonomia tradicional como *Herpetomonas* ficam posicionados em clados distantes e não relacionados (figura 2). Sendo assim, o gênero *Herpetomonas* é atualmente considerado polifilético.

Diversos marcadores moleculares têm sido utilizados na tentativa de distinguir as espécies de *Herpetomonas*. Análises de polimorfismos de restrição e hibridização cruzada usando sequências de DNA ribossomal e de kDNA, além de distinguir diferentes espécies de *Herpetomonas* revelaram que espécies anteriormente classificadas neste gênero são mais relacionadas com outros gêneros de tripanossomatídeos (Camargo et al., 1992;. Nunes et al., 1994;. Teixeira et al., 1997). Análises de padrões de RAPD e de RFLP revelaram polimorfismos intraespecíficos no gênero *Herpetomonas* (Fiorini et al., 2001).

Recentemente, a combinação de dados filogenéticos e morfológicos foi utilizada na descrição de novas espécies de *Herpetomonas* encontradas em duas espécies de Diptera pertencentes à familia Ceratopogonidae: *Herpetomonas ztiplika* isolada de *Culicoides kibunensis* (Podlipaev et al., 2004a) e *Herpetomonas trimorpha*, isolada de *Culicoides truncorum* (Zídková et al., 2010). Embora existam inúmeras descrições de *Herpetomonas* spp. na literatura, somente uma pequena parte foi cultivada e está disponível para estudos comparativos (Figura 6).

**A** **B** **C**

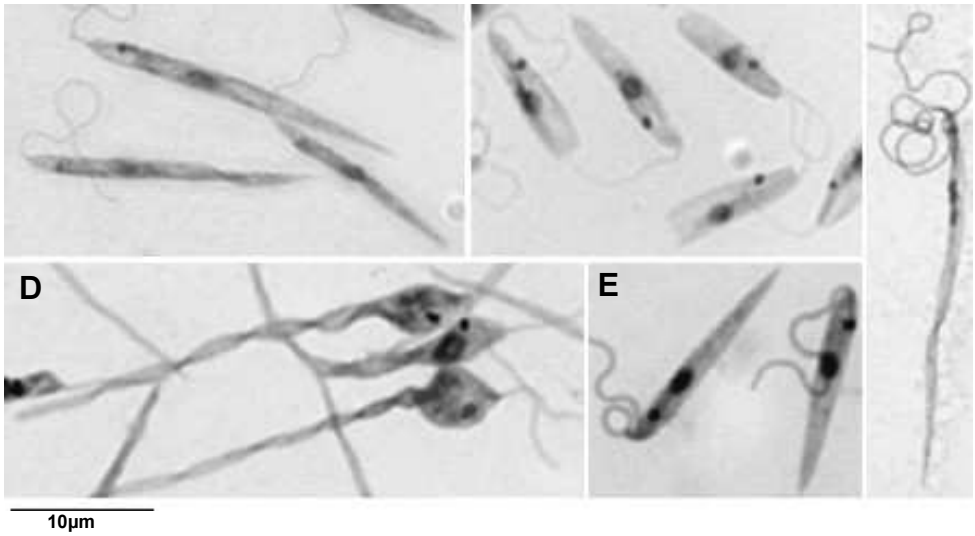


**Figura 6**: Microscopia de luz de espécies de *Herpetomonas*: A) *H. muscarum*; B) *H. samuelpessoai*; C) *H. trimorpha* (modificada de Zídková et al., 2010); D) *H. mirabilis,* E) *H. mariadeanei.* Note o acentuado polimorfismo encontrado entre as espécies no gênero *Herpetomonas*.

## 1.6 Genes utilizados para filogenia e taxonomia de tripanossomatídeos

A taxonomia dos tripanossomatídeos baseada em parâmetros taxonômicos tradicionais tem se mostrado incompatível com evidências demonstradas por dados moleculares. Embora estudos atuais apontem para a eficácia do uso de marcadores moleculares para gerar hipóteses evolutivas filogenéticas confiáveis da família Trypanosomatidae, os estudos focam parasitas monoxênicos e esses empregam um número pequeno de espécies. Desde as primeiras análises filogenéticas baseadas em sequências de genes ribossômicos (Sogin et al., 1986; Fernandes et al., 1993), o uso de sequências gênicas tem sido valioso na reconstrução da história evolutiva desses organismos.

Marcadores moleculares têm sido utilizados por diversos grupos, apesar de muitas vezes os resultados não serem facilmente comparáveis. Entretanto, esse é um requisito indispensável na taxonomia atual.

### 1.6.1 Gene ribossômico

Sequências dos genes ribossômicos têm sido amplamente utilizadas para inferir relações filogenéticas entre espécies do filo Euglenozoa. Os tripanossomatídeos possuem um dos mais complexos padrões de moléculas

maduras de RNA. Os genes de RNA ribossômico (rRNA) consistem de unidades de repetição compostas por unidades de transcrição (cistrons ribossômicos) que são intercaladas por espaçadores intergênicos (IGS) e se repetem em tandem mais de 100 vezes no genoma. Estes genes são processados em uma única unidade de transcrição conhecida como pré-rRNA. Após várias etapas de processamento o pré-rRNA dá origem a três moléculas de RNA maduros: 18S (SSU ou subunidade menor), 5.8S e 24S (LSU ou subunidade maior), que nestes organismos é constituída por dois fragmentos de alto peso molecular (24Sα e 24Sβ) e quatro subunidades de rRNAs de baixo peso molecular (S1, S2, S4 e S6). As subunidades SSU e LSU são constituídas por sequências altamente conservadas e intercaladas por espaçadores de conservação intermediária ITS (ITS 1 e 2, espaçadores internos transcritos) e ETS (espaçador externo transcrito) que são flanqueados pelo espaçador intergênico (IGS), que apresenta sequências altamente variáveis (Sogin et al., 1986; Hernández et al., 1990) (Figura 7).

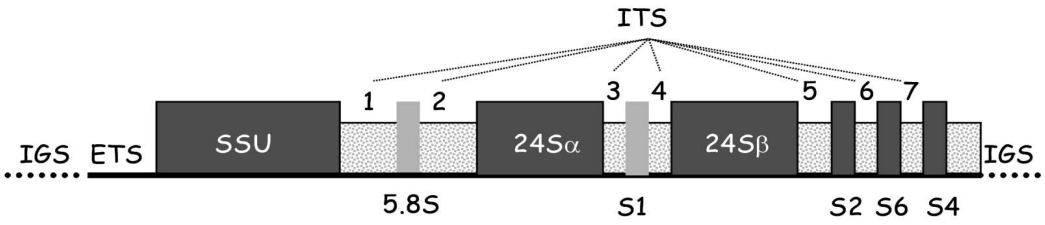


**Figura 7**. Representação esquemática do cistron ribossômico de tripanossomatídeos.

Esses genes são utilizados para inferências de relacionamentos filogenéticos, pois são funcionalmente equivalentes em todos os organismos e apresentam domínios com diferentes graus de conservação (Sogin et al., 1986; Hernández et al., 1990). A presença de diversas regiões, transcritas ou não, que exibem diferentes graus de conservação, faz desses genes excelentes alvos para identificação de gêneros, espécies, linhagens e genótipos (Souto et al., 1996, Zingales et al., 1998; Brisse et al., 2001; Stevens et al., 2001; Maia da Silva et al., 2004b; Hamilton et al., 2004, 2007; Rodrigues et al, 2006; Cortez et al., 2006; Ferreira et al., 2007, 2008; Viola et al., 2008, 2009a,b). Além disso, o polimorfismo de sítios de enzimas de restrição nos genes ribossômicos de tripanossomatídeos também pode auxiliar na

classificação em gêneros. Já foi demonstrado que espécies de *Crithidia* que não tem simbiontes (apossimbióticas) e *Herpetomonas* tem sítios para *PvuII* na SSU, ausentes em *Phytomonas*. Um sítio para *Hind* III, observado na LSU (24Sα) das espécies de *Crithidia* apossimbióticas não foi detectado em *Phytomonas* e *Herpetomonas.* As espécies de *Leptomonas* e os tripanossomatídeos com endossimbontes apresentaram heterogeneidade destes sítios de restrição (Camargo et al., 1992; Teixeira et al., 1994;1997; Batistoti et al., 2001). Em análises por "riboprinting" de SSUrDNA amplificado por PCR de 19 espécies/isolados do gênero *Crithidia* revelou-se uma grande variabilidade genética entre os isolados analisados, que foram separados em 7 tipos/grupos: 1) *C. deanei*; 2) *C. oncopelti*; 3) *C. fasciculata* e *C. luciliae*; 4) *C. flexonema*; 5) *C. melificae*; 6) *C. acanthocephali* e *C. harmosa*; 7) *C. hutneri* e *C. l. thermophila* (Clark, 1997).

As sequências do gene SSU rRNA são as mais utilizadas em estudos comparativos devido a características importantes, tais como: a) o pequeno tamanho que permite fácil obtenção por amplificação por PCR; b) a presença de regiões variáveis flanqueadas por regiões conservadas que permitem alinhamentos altamente confiáveis, com oito regiões universalmente conservadas (U1-U8) e nove regiões variáveis (V1-V9) (Hernández et al., 1990). Além disso, existem dezenas de sequências de SSU rRNA de diferentes espécies de flagelados da família Trypanosomatidae depositadas em bancos de sequências, permitindo a rápida comparação de novas sequências com as de espécies ou linhagens já conhecidas.

Os espaçadores IGS e ITS são muito mais variáveis que as regiões SSU e LSU. A região entre a SSU e a LSU contêm três regiões: ITS1, 5.8S altamente conservada) e ITS2. As sequências de ITS1 e ITS2 diferem inter e intra-especificamente, sendo excelentes para análises de organismos filogeneticamente próximos assim como alvos para diagnóstico.

Análises do tamanho e de sítios de restrição de ITS rDNA, especialmente ITS1, revelaram-se valiosas no estudo de variabilidade inter e intraespecificas de flagelados dos gêneros *Trypanosoma* (Cuervo et al., 2002; Fernandes et al., 1999; Ferreira et al., 2007; Lima et al., 2012; Maia da Silva et al., 2004b; Rodrigues et al., 2006, 2008; Santos et al., 2002) e *Leishmania* (Cupolillo et al, 1995; 1998; Schönian et al., 2000). Resultados obtidos por nosso grupo mostraram que tamanhos de ITS separam grupos intraespecíficos de *Herpetomonas* (Teixeira et al., 1996; 1997;

Takata et al., 1996; Fiorini et al., 2001) e de *Phytomonas* (Serrano, 2000). Recentemente Dollet e colaboradores (2012), baseados em sequências de ITS rDNA, identificaram 10 grupos distintos de isolados de *Phytomonas*, evidenciando, assim, a utilidade de sequências de ITS rDNA para avaliar a diversidade de tripanossomatídeos de plantas.

### 1.6.2 Gene codificador da enzima Gliceraldeído 3-fosfato desidrogenase glicossômica (gGAPDH)

Nas espécies da família Trypanosomatidae, as enzimas envolvidas no metabolismo de glicose e glicerol encontram-se em organelas peculiares, chamadas glicossomos. A compartimentalização de enzimas da via glicolítica em tripanossomatídeos contrasta com sua localização citossólica nos demais eucariotos. Em tripanossomas, foram encontrados dois genes que codificam a enzima glicossômica (gGAPDH) e um gene que codifica uma enzima citosólica (cGAPDH). (Michels et al. 1986; Kendall et al. 1990) (Figura 8).


**Figura 8**. Representação esquemática dos genes de GAPDH.

Os genes de gGAPDH apresentam duas cópias praticamente idênticas e por codificarem proteínas, estão sujeitos a diferentes pressões seletivas e taxas de evolução se comparados aos genes ribossômicos. Os genes de gGAPDH são excelentes marcadores para estudos filogenéticos de tripanossomatídeos, permitindo alinhamentos confiáveis de sequências de organismos geneticamente distantes (Hamilton et al., 2004, 2005a,b, 2007; Stevens, 2008).

Estudos filogenéticos utilizando sequências de gGAPDH e SSU rRNA de um grande número de espécies de tripanossomatídeos geraram topologias congruentes e análises independentes e combinadas desses genes têm sido recomendadas na descrição de gêneros, subgêneros e espécies de tripanossomatídeos (Hamilton et al., 2004, 2005a, 2009; Viola et al., 2009b; Maslov et al., 2010). Um estudo realizado com um grande número de tripanossomatídeos baseado em genes gGAPDH e que

utilizou diferentes métodos de inferências filogenéticas, confirmou a polifilia do gênero *Crithidia*, assim como a monofilia dos tripanossomatídeos com endossimbiontes (Hamilton et al., 2004).

### 1.6.3 Polimorfismo de moléculas de minicírculos de kDNA

Os cinetoplastídeos modificaram sua única mitocôndria, alterando o conteúdo de DNA e sua organização, gerando o cinetoplasto, uma região rica em moléculas de DNA (kDNA) concatenadas em uma única rede. O kDNA consiste de moléculas dupla-fita circulares possuindo entre 5.000-10.000 minicírculos e 20-40 maxicírculos (Englund, 1978; Marini et al., 1980; Lukeš e Votýpka, 2000; Jensen e Eglund, 2012).

Assim como DNA mitocondrial de outros organismos, os maxicírculos codificam rRNA e várias proteínas envolvidas na transdução de energia (incluindo subunidades de citocromo-oxidase, NADH desidrogenase e ATP - sintetase). Já os minicírculos codificam os RNAs guias, que participam do processo de edição das moléculas de RNA codificadas nos maxicírculos (Jensen e Eglund, 2012).

Os minicírculos do kDNA dos tripanossomatídeos apresentam tamanhos muito variáveis e sequências heterogêneas, exceto pela presença de uma região conservada formada por ~100-200 pares de bases chamada UMS (“Universal Minicircle Sequence”) que corresponde à origem de replicação. Essa região é formada por três blocos CSB ("Conserved Sequence Blocks") - CSBs 1, 2 e 3 - e dependendo as espécie pode ter uma, duas ou quatro cópias com localizações opostas e distantes simetricamente (Jensen and Englund, 2012; Simpson, 1987; Lukeš et al., 2002; Ray, 1989). *Crithidia fasciculata,* uma das espécies mais utilizadas em estudos de organização e replicação de kDNA (Guilbride e Englund, 1998; Morris et al., 2001; Klingbeil e Englund, 2004), apresenta duas UMS separadas a 180° (Figura 9) (Jensen e Englund, 2012; Sugisaki e Ray, 1987).

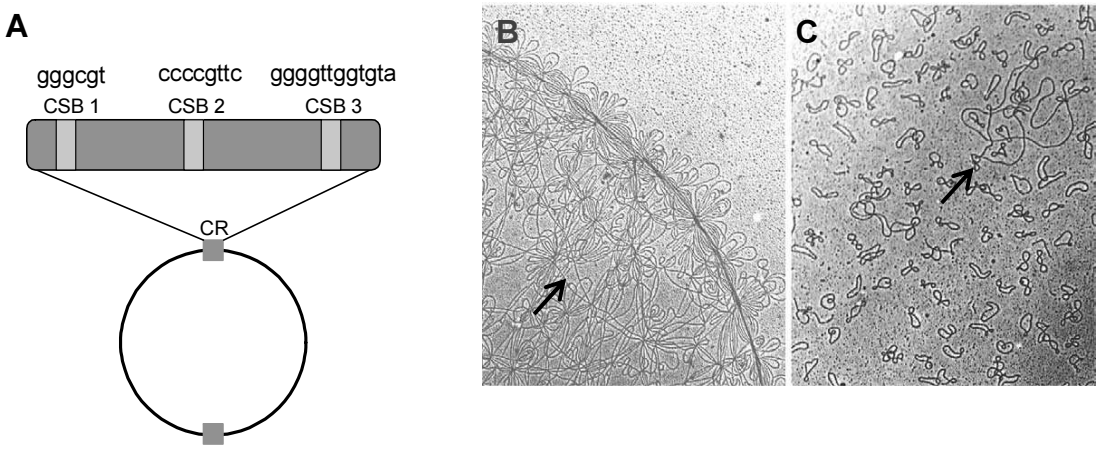


**Figura 9**. A: Representação esquemática da organização das moléculas de minicírculos de kDNA de *C. fasciculata:* CR, Conserved region; CSB, "Conserved Sequence Blocks" (Ray, 1989). B: Microscopia eletrônica da rede de kDNA de *C. fasciculata,* e C: kDNA desconcatenados. As setas indicam os maxicírculos (modificada de Liu et al., 2005)

Interespecificamente os minicírculos do kDNA diferem acentuadamente em tamanho e sequência. Intraespecificamente, estas moléculas diferem na sequência, mas são, geralmente, homogêneas em tamanho, ou pelo menos apresentam uma classe majoritária. O polimorfismo de tamanho de minicírculos do kDNA varia conforme o gênero; o maior é o encontrado entre espécies de *Trypanosoma*, que varia de 465bp em *T. vivax* (Borst et al., 1985) a mais de 5,0kb em tripanossomas de peixes (Jirkůu et al., 1995) e até ~10,0kb em *T. avium* (Yurchenko et al., 1999; Lukeš e Votýpka, 2000). As espécies de *Leishmania* apresentam uma pequena variação de tamanho de minicírculos (~600 a 970bp), que são heterogêneos em sequências (Yurchenko e Kolesnikov, 2001).

As análises de minicírculos de kDNA revelaram um só tamanho por espécie do gênero *Phytomonas*, grande heterogeneidade entre as espécies, com pelo menos cinco classes de moléculas (tamanhos de 1,3 a 2,8kb), porém, com grupos de isolados que compartilham o tamanho destas moléculas (Muller et al., 1995). O sequenciamento de minicírculos de *P. serpens* revelou duas origens de replicação (Dollet et al., 2001; Sá-Carvalho et al., 1993; Maslov et al., 1998).

Ao contrário das espécies dos gêneros *Herpetomonas*, *Leptomonas* e *Phytomonas*, que apresentam minicírculos que variam de tamanho e de sequência apenas interespecificamente, os membros do gênero *Crithidia* apresentam classes de tamanhos e sequências variáveis intraespecificamente.

Pestov e colaboradores (1990) descreveram quatro classes de minicírculos (1,3; 1,6: 1,9 e 2,2 kb) em *C. oncopelti*. Posteriormente, foi demonstrado que o tamanho dos minicírculos de kDNA permite agrupar os isolados do gênero *Crithidia* e que as espécies com endossimbiontes apresentam minicírculos muito heterogêneos quanto ao tamanho (entre 1,3 e mais de 4.0kb) e número de classes. As espécies com endossimbiontes apresentaram três padrões de moléculas representadas, respectivamente por: *C. deanei, C. desouzai* e *C. oncopelti*. Os tripanossomatídeos com formas coanomastigotas sem endossimbiontes foram distribuídos em três grupos representados respectivamente por *C. fasciculata C. l. termophila* e *C. acantocephali* (Teixeira et al., 1995, Malvezzi et al. 1996). Hollar e colaboradores (1998) também mostraram que os minicírculos de algumas espécies com endossimbiontes apresentam tamanhos acima de 4,0kb, sendo esses os maiores encontrados entre tripanossomatídeos de insetos. A heterogeneidade e o agrupamento de espécies com formas coanomastigotas, com base no tamanho dessas moléculas foram confirmados por Brandão et al., (2000).

Devido ao grande número de cópias e a divergência entre espécies, os minicírculos de kDNA têm sido alvos de métodos diagnósticos. A homogeneidade de tamanho das moléculas tem sido explorada para o diagnóstico de *T. cruzi* por PCR (Ávila et al., 1990; Wincker et al., 1994; Junqueira et al., 1996; Gomes et al., 1998) e para separar esta espécie de *T. rangeli* (Vallejo et al., 1999). A amplificação de sequência de minicírculos de kDNA também tem sido bastante utilizada no diagnóstico de *Leishmania* spp. (Noyes et al., 1998; Martin Sanches, 2004; Cortes et al., 2004). Recentemente, de Pereira e Brandão (2013) demonstrarm que o perfil de composição de dinuclotideos dos minicirculos podem ser capazes de diferenciar membros pertencentes a familia Trypanossomatidae.

Os padrões de digestão de kDNA por endonucleases de restrição (esquizodemas) são característicos para espécies de tripanossomatídeos (Morel e Simpson, 1980; Morel et al., 1986; Camargo et al., 1982). Diferentes espécies de *Crithidia* diferem nos padrões de esquizodema (De Sa et al., 1980; Camargo et al., 1982; Hollar et al., 1998). A heterogeneidade intraespecífica de sequências de kDNA foi utilizada para identificar subgrupos de *Herpetomonas* (Nunes et al., 1994; Teixeira et al., 1997; Fiorini et al., 2001). Grupos de isolados de *Phytomonas* definidos de acordo com o tamanho destas moléculas foram confirmados por

marcadores de RAPD e por sequências dos genes ribossômico e SL (Serrano, 2000).

## 2. JUSTIFICATIVA E OBJETIVOS

Tripanossomatídeos parasitas de insetos são os mais abundantes na família e tem sido encontrados em mais de 400 espécies de insetos em todo mundo. Seus hospedeiros preferenciais são hemípteros e dípteros onde se desenvolvem no trato digestivo. Geralmente não são patogênicos para o hospedeiro e são transmitidos para outros insetos através de contaminação, coprofagia ou predação.

Tradicionalmente, a taxonomia da família Trypanosomatidae tem sido baseada em parâmetros morfológicos, ciclo de vida e hospedeiros de origem. Entretanto, estudos filogenéticos recentes baseados em diversos marcadores moleculares não têm corroborado grande parte da taxonomia tradicional, questionando os gêneros e muitas das espécies descritas de tripanossomatídeos. Esses estudos apoiam a monofilia dos gêneros *Trypanosoma*, *Leishmania* e *Phytomonas*. Entretanto, os demais gêneros de tripanossomatídeos têm se apresentado como não monofiléticos mostrando a insuficiência da taxonomia tradicional para definir esses gêneros.

Os tripanossomatídeos de insetos apresentam baixa especificidade com o hospedeiro e uma grande diversidade morfológica. Nos últimos anos, o uso da morfologia juntamente com métodos baseados em marcadores moleculares e análises filogenéticas baseadas em genes como spliced leader, 5S rRNA, SSU rRNA e gGAPDH vem contribuindo para o entendimento da biodiversidade e filogenia de organismos da família Trypanosomatídae.

Muitos tripanossomatídeos monoxênicos foram descritos parasitando moscas de diversas espécies e famílias. Entretanto, poucos estudos foram realizados a fim de classificar esses flagelados. Estudos sobre tripanossomatídeos de dípteros de diversas subordens e famílias têm sido de grande valor para melhor entender a história evolutiva dos tripanossomatídeos em geral. Estudos preliminares sugerem que moscas da família Calliphoridae e Muscidae albergam uma grande quantidade e diversidade de tripanossomatídeos. As moscas de família Calliphoridae são excelentes hospedeiros para estudos evolutivos da família Trypanosomatidae devido a diversos fatores: existência no Brasil de espécies endêmicas assim como originárias da Europa e África introduzidas recentemente (*Chrysomya* sp.); presença de espécies que habitam ecótopos diferentes e podem ser ou não sinantrópicas;

disponibilidade de dados filogeográficos, entre outros. Nos últimos anos análises filogenéticas tem sido muito utilizada para reclassificar e criar novas espécies e gêneros de flagelados encontrados em dípteros dentro da família Trypanosomatídae.

Deste modo este estudo tem como objetivo:

1) Analisar a ocorrência de tripanossomatídeos em dípteros capturados em diferentes regiões e países da América do Sul (Brasil, Colômbia e Venezuela) e do continente africano (Moçambique, Guiné Bissau e Madagascar);

2) Avaliar a diversidade morfológica dos isolados obtidos por análises de microscopia de luz e eletrônica;

3) Classificar os isolados por análise de sequências da região variável V7-V8 SSU rDNA (barcoding) e selecionar representantes de todas as espécies e genótipos encontrados;

4) Inferir relações filogenéticas e filogeográficas baseadas nos genes SSU rRNA e *g*GAPDH entre os flagelados isolados de dípteros obtidos neste estudo com os demais tripanossomatídeos de dípteras já descritos;

5) Avaliar o polimorfismo inter- e intra-específico com a utilização de marcadores polimórficos como: sequencias de ITS1 rDNA e polimorfismo de tamanho e de número de classes de minicírculos de kDNA.

# 3. MATERIAL E MÉTODOS

## 3.1 Captura dos dípteros

A captura dos dípteros foi feita por busca ativa ou com armadilhas utilizando iscas tais como: fígado, frutos de aromas fortes ou em decomposição. As moscas foram capturadas na América do Sul (Brasil, Colômbia e Venezuela) e no continente africano (Guiné Bissau, Moçambique e Madagascar). Os dípteros positivos quanto a presença de tripanossomatídeos foram mantidos em etanol 100% para posterior identificação. A identificação dos dípteros brasileiros e africanos foi feita baseando-se em chaves de identificação realizadas por: Patricia J. Thyssen, Silvio Nihei, Priscylla Moll Arruda e Ana Maria Lima de Azeredo-Espin.

## 3.2 Organismos

Os organismos utilizados neste estudo e seus respectivos hospedeiros de origem e local de isolamento estão relacionados na tabela 1. Todas as culturas obtidas neste estudo estão criopreservadas na coleção de tripanossomatídeos do Departamento de Parasitologia, TCC/USP (Trypanosomatid Culture Collection). Os isolados são mantidos congelados em $N_2$ líquido (em meio LIT com 10% de SFB acrescido de 20% de DMSO).

## 3.3 Isolamento e cultivo de tripanossomatídeos de insetos

Os tubos digestivos (TDs) das moscas foram dissecados e investigados a fresco, por microscopia de luz, quanto à presença de tripanossomatídeos. Os tratos digestivos infectados com tripanossomatídeos foram utilizados para confecção de lâminas e inoculados em meio bifásico composto por uma fase sólida a 4% de "Blood Agar Base" (BAB) acrescido de 15% de sangue de coelho e a fase líquida composta por LIT (Liver Infusion Tryptose) (Camargo, 1964) e acrescido de 10% de soro fetal bovino (SFB). Para eliminar eventuais contaminações das amostras por bactérias foi adicionado 80µg/ml de gentamicina e 1mg/ml de ampicilina na fase

líquida. Alguns tubos digestivos foram também preservados em etanol 100%, para posterior análise. Quando as culturas estavam contaminadas com leveduras, os tripanossomatídeos eram separados por passagens em tubo em U. Os meios de cultura utilizados estão descritos na Tabela 2.

Tabela 1. Isolados de trupanossomatídeos de dípteros utilizados neste estudo

| Organismo | TCC | Hospedeiro | | Origem Geografica | |
|---|---|---|---|---|---|
| | | Família | espécie | Cidade/estate | Pais |
| ***A. desouzai*** | | | | | |
| Genótipo Ads1 | 079E | Sy. | *Ornidia obesa* | Alfenas/MG | Brasil |
| | 1279 | Ca. | *Lucilia eximia* | Campo Grande/MS | Brasil |
| | 1310 | Ca. | *Lucilia eximia* | Campo Grande/MS | Brasil |
| | 1311 | Ca. | *Lucilia eximia* | Campo Grande/MS | Brasil |
| | 1312 | Ca. | *Lucilia eximia* | Campo Grande/MS | Brasil |
| | 1429 | Ca. | *Lucilia eximia* | Campo Grande/MS | Brasil |
| | 1453 | Ca. | *Lucilia eximia* | Campo Grande/MS | Brasil |
| | 2214 | Ca. | *Lucilia eximia* | Miranda/MS | Brasil |
| | 2459 | Ca. | *Crysomya* sp | São Carlos/SP | Brasil |
| | 2460 | Ca. | *Crysomya* sp | São Carlos/SP | Brasil |
| | ISC0010a | Ca. | *Crysomya* sp | Mérida/Mda | Venezuela |
| | ISC0012a | Ca. | *Crysomya* sp | Potrerillo/Tol | Colômbia |
| | ISC0016b | Ca. | *Crysomya* sp | Mérida/Mda | Venezuela |
| | ISC0001c | Sa. | | Chupanga/Sofala | Moçambique |
| | ISC0022a | Sa. | | Zulia | Venezuela |
| Genótipo Ads 2 | | | | | |
| | 2317 | Mu. | Musca sp | Marromeu/Sofala | Moçambique |
| ***A. ambiguus*** | | | | | |
| | 1780 | Ca | *Chrysomya albiceps* | Campo Grande/MS | Brasil |
| | 1765 | Ca | *Chrysomya putoria* | Buba/Tchingue | Guiné Bissau |
| | ISC0004a | Ca. | *Chrysomya putoria* | Antsitanana | Madagascar |
| | ISC0010 | Ca. | *Crysomya* sp | Mérida/Mda | Venezuela |
| | ICS0011 | Ca. | *Crysomya* sp | Potrerillo/Tol | Colômbia |
| | ISC0013b | Ca. | *Crysomya* sp | Ortega/Tol | Colômbia |
| ***A. deanei*** | | | | | |
| Genótipo Ade1 | 036E | Red. | *Zelus. leucogrammus* | Goiânia/GO | Brasil |
| | 263E | Ca. | *Chrysomya putoria* | Rolândia/PR | Brasil |
| | 080E | Syr. | *Ornidia obesa* | Alfenas/MG | Brasil |
| | 1158 | Syr | *Ornidia obesa* | São Paulo/SP | Brasil |
| | 1503 | Ca. | *Chrysomya albiceps* | Campo Grande/MS | Brasil |
| | 1504 | Ca. | *Chrysomya albiceps* | Campo Grande/MS | Brasil |
| | 1505 | Ca. | *Chrysomya albiceps* | Campo Grande/MS | Brasil |
| | 1743 | Ca. | *Lucilia cuprina* | São Paulo/SP | Brasil |
| | 1752 | Ca. | *Lucilia cuprina* | São Paulo/SP | Brasil |
| | 1757 | Ca. | *Chrysomya putoria* | São Paulo/SP | Brasil |
| | 2315 | Ca. | *Chrysomya putoria* | Chupanga/Sofala | Moçambique |
| | 2314 | Mu. | *Musca* sp | Marromeu/Sofala | Moçambique |
| | 2316 | Ca. | *Chrysomya putoria* | Chupanga/Sofala | Moçambique |
| | ISC0001b | Sa. | | Chupanga/Sofala | Moçambique |
| Genótipo Ade2 | | | | | |
| | 1759 | Ca. | *Chrysomya putoria* | Buba/Tchingue | Guiné Bissau |
| | 2332 | Mu. | *Musca* sp | Marromeu/Sofala | Moçambique |
| | 2317 | Mu. | Musca sp | Marromeu/Sofala | Moçambique |
| | 2455 | Mu. | *Musca* sp | Ortega/TOL | Colômbia |
| Genótipo Ade3 | | | | | |
| | 1267 | Ca. | *Chrysomya putoria* | Maputo/MPT | Moçambique |
| | 1427 | Ca. | *Chrysomya albiceps* | Campo Grande/MS | Brasil |

| | | | | | |
|---|---|---|---|---|---|
| | 1445 | Ca. | *Chrysomya megacephala* | Campo Grande/MS | Brasil |
| | 1447 | Ca. | *Lucilia eximia* | Campo Grande/MS | Brasil |
| | 1639 | Ca. | *Chrysomya magacephala* | Chupanga/Sofala | Moçambique |
| | 1675 | Ca. | *Chrysomya putoria* | Maputo/MPT | Moçambique |
| | 1715 | Ca. | *Lucilia* sp | Bastos/SP | Brasil |
| | 1742 | Ca. | *Chrysomya megacephala* | Monte Negro/RO | Brasil |
| | 1754 | Ca. | *Chrysomya magacephala* | Monte Negro/RO | Brasil |
| | 1755 | Ca. | *Lucilia cuprina* | Monte Negro/RO | Brasil |
| | 1756 | Ca. | *Lucilia cuprina* | Monte Negro/RO | Brasil |
| | 1758 | Ca. | *Chrysomya magacephala* | Monte Negro/RO | Brasil |
| | 1760 | Ca. | *Chrysomya putoria* | Buba/Tchingue | Guiné Bissau |
| | 1762 | Ca. | *Chrysomya putoria* | Buba/Tchingue | Guiné Bissau |
| | 1763 | Ca. | *Chrysomya putoria* | Buba/Tchingue | Guiné Bissau |
| | 1884 | Ca. | *Chrysomya magacephala* | Confresa/MT | Brasil |
| | 1920 | Sa. | | Gorongosa/Sofala | Moçambique |
| | 1923 | Ca. | *Chrysomya putoria* | Chupanga/Sofala | Moçambique |
| | 1940 | Ca. | *Cochliomyia macellaria* | Miranda/MS | Brasil |
| | 2018 | Ca. | *Chrysomya putoria* | Cufada/Buba | Guiné Bissau |
| | 2025 | Ca. | *Chrysomya putoria* | Cufada/Buba | Guiné Bissau |
| | 2046 | Ca. | *Chrysomya magacephala* | Campo Grande/MS | Brasil |
| | 2052 | Ca. | *Chrysomya magacephala* | Campo Grande/MS | Brasil |
| | 2054 | Ca. | *Chrysomya albiceps* | Campo Grande/MS | Brasil |
| | 2065 | Ca. | *Chrysomya magacephala* | Campo Grande/MS | Brasil |
| | 2066 | Ca. | *Chrysomya magacephala* | Campo Grande/MS | Brasil |
| | 2067 | Ca. | *Chrysomya magacephala* | Campo Grande/MS | Brasil |
| | 2068 | Ca. | *Chrysomya magacephala* | Campo Grande/MS | Brasil |
| | 2070 | Ca. | *Chrysomya albiceps* | Campo Grande/MS | Brasil |
| | 2093 | Ca. | *Chrysomya albiceps* | Nhecolandia/MS | Brasil |
| | 2313 | Gl. | *Glossina* sp | Chupanga | Moçambique |
| | 2454 | Sa. | | Araguaina/TO | Brasil |
| | 2534 | Ca. | *Chrysomya* sp | Moramanga | Madagascar |
| | 2554 | Mu. | *Musca* sp | Moramanga | Madagascar |
| | 2601 | Ca. | *Chrysomya* sp. | Moramanga | Madagascar |
| | ISC0004b | Ca. | *Chrysomya putoria* | Madagascar | Madagascar |
| | ISC0010a | Ca. | *Chrysomya* sp. | Mérida/Mda | Venezuela |
| | ISC0011a | Ca. | *Chrysomya* sp. | Potrerillo/Tol | Colômbia |
| | ISC0013c | Ca. | *Chrysomya* sp. | Ortega/Tol | Colômbia |
| Genótipo Ade4 | | | | | |
| | 2446 | Mu. | *Musca* sp | Payandé/TOL | Colômbia |
| | 2447 | Mu. | *Musca* sp | Payandé/TOL | Colômbia |
| | 2448 | Mu. | *Musca* sp | Payandé/TOL | Colômbia |
| | 2449 | Mu. | *Musca* sp | Payandé/TOL | Colômbia |
| | 2450 | Mu. | *Musca* sp | Payandé/TOL | Colômbia |
| | 2453 | Mu. | *Musca* sp | Payandé/TOL | Colômbia |
| ***H. muscarum*** | | | | | |
| | 001E | Mu. | *Musca domestica* | Minneapolis/MN | Estados Unidos |
| | 002E[a] | Ph. | *Megaselia scalaris* | Lincoln/NE | Estados Unidos |
| | 251E | Mu. | *Musca domestica* | Itapetininga/SP | Brasil |
| | 257E | Mu. | *Musca domestica* | Itapetininga/SP | Brasil |
| | 261E | Mu. | *Musca domestica* | Arapongas/PR | Brasil |
| | 284E | Sy. | *Ornidia obesa* | Itapetininga/SP | Brasil |
| ***H. samuelpessoai*** | | | | | |
| | 264E | Fa. | *Fannia canicularis* | Rolândia/PR | Brasil |
| | 281E | Sa. | *Sarcophaga sp.* | São Paulo/SP | Brasil |
| | 282E | Sa. | *Sarcophaga sp.* | São Paulo/SP | Brasil |
| | 1443 | Ca. | *Chrysomya megacephala* | Campo Grande/MS | Brasil |
| | 1545 | Mu. | *Musca domestica* | Rolândia/PR | Brasil |
| | 1716 | Ca. | *Lucilia* sp. | Bastos/SP | Brasil |
| | 1761 | Ca. | *Chrysomya putoria* | Cufada | Guiné Bissau |
| | 1851 | Ca. | *Chrysomya putoria* | Cufada | Guiné Bissau |
| | 1861 | Sa. | *Peckia ingens* | São Paulo/SP | Brasil |
| | 1870 | Sa. | *Peckia ingens* | São Paulo/SP | Brasil |

| | | | | | |
|---|---|---|---|---|---|
| ***H. isaaci*** | | | | | |
| | 266E | Mu. | *Musca domestica* | Rolândia/PR | Brasil |
| | 270 | Ca. | *Chrysomya albiceps* | Alfenas/MS | Brasil |
| | 276E | Ca. | *Chrysomya putoria* | Bastos/SP | Brasil |
| | 278E | Ca. | *Chrysomya megacephala* | São Paulo/SP | Brasil |
| | 1533 | Mu. | *Musca domestica* | Rolândia/PR | Brasil |
| | 1699 | Ca. | *Chrysomya putoria* | Bastos/SP | Brasil |
| | 1724 | Sy. | *Ornidia obesa* | Monte Negro/RO | Brasil |
| | 1766 | Ca. | *Chrysomya putoria* | Cufada | Guiné Bissau |
| | 1767 | Ca. | *Chrysomya megacephala* | Monte Negro/RO | Brasil |
| | 1881 | Ca. | *Chrysomya megacephala* | Confresa/MT | Brasil |
| | 1882 | Ca. | *Chrysomya megacephala* | Canfresa/MT | Brasil |
| | 1885 | Ca. | *Chrysomya megacephala* | Confresa/MT | Brasil |
| | 1852 | Ca. | *Chrysomya albiceps* | São Paulo/SP | Brasil |
| | 1941 | Ca. | *Lucilia eximia* | Miranda/MS | Brasil |
| | 1944 | Ca. | *Chrysomya megacephala* | Miranda/MS | Brasil |
| | 2026 | Ca. | *Chrysomya putoria* | Cufada | Guiné Bissau |
| | 2069 | Ca. | *Chrysomya megacephala* | Campo Grande/MS | Brasil |
| ***H. puelarum*** | | | | | |
| | 1745 | Ca. | *Chrysomya putoria* | Cufada | Guiné Bissau |
| | 247E | Mu. | *Musca domestica* | Cotia/SP | Brasil |
| | 254E | Mu. | *Musca domestica* | Itapetininga/SP | Brasil |
| ***H. elegans*** | | | | | Brasil |
| | 1733 | Sy. | *Ornidia obesa* | Monte Negro/RO | Brasil |
| | 1727 | Sy. | *Ornidia obesa* | Monte Negro/RO | Brasil |
| | 1848 | Sy. | *Ornidia obesa* | São Paulo/SP | Brasil |
| ***H. modestus*** | | | | | Brasil |
| | 1444 | Ca. | *Chrysomya megacephala* | Campo Grande/MS | Brasil |
| | 1943 | Ca. | *Cochliomyia macellaria* | Miranda/MS | Brasil |
| | 2049 | Ca. | *Chrysomya megacephala* | Campo Grande/MS | Brasil |
| ***H. mirabilis*** | 301E | Ca. | *Cynomyopsis cadaverina* | Chichicastenango/El Quiché | Guatemala |
| ***H. wanderleyi*** | 1982 | Ca. | *Cochliomyia macellaria* | Miranda/MS | Brasil |
| ***H. mariadeanei*** | 004E | Mu. | *Muscina stabulans* | São Paulo/SP | Brasil |

Familias dos insetos: Ca, Calliphoridae; Fa, Fanniidae; Mu, Muscidae; Ph, Phoridae; Sa, Sarcophagidae; Sy, Syrphidae
TCC ( truypanosomatid culture collection; ISC (Insect sample collection)

### 3.4 Investigação da presença de endossimbiontes bacterianos

Para detectar a presença de endossinbiontes bacterianos nos isolados analisados, tripanossomatídeos foram cultivados em meio LIT ou FYTS não suplementado com hemina, incubados a 28$^{o}$C por mais de 20 dias, com repiques sucessivos de dois em dois dias. Para confirmar a presença de endossinbiontes, as culturas que apresentaram desenvolvimento com as condições descritas acima foram ampliadas, submetidas à extração de DNA e empregadas em ensáios de PCR com oligonucleotídeos específicos para amplificar um fragmento de ~600bp do gene 16S rRNA de bactérias.

**Tabela 2. Meios de cultura utilizados para o cultivo dos tripanossomatídeos utilizados neste estudo.**

| Meio de cultura | Preparo e Armazenamento | Composição | Quantidade/ Litro |
|---|---|---|---|
| LIT[(1)] | Esterilizado em autoclave e armazenado a -20°C. | Triptose | 5,00g |
| | | Infuso de fígado | 5,00g |
| | | NaCl | 4,00g |
| | | KCl | 0,40g |
| | | $Na_2HPO_4$ | 8,00g |
| | | Glicose | 2,00g |
| | | Hemina[a] | 0,01g |
| | | SFB (soro fetal bovino)[b] | 100mL |
| BABb/LIT [(2)] | Armazenado a 4°C | Meio BAB (blood Agar base) a 4% | 850mL |
| | | Sangue de coelho estéril e desfibrinado | 150mL |
| FYTS[(3)] | Esterilizado em autoclave e armazenado a -20°C. | Sacarose | 20g |
| | | Bactriptona | 5g |
| | | Extrato de levedura | 5g |
| | | Ácido fólico | 0,02g |
| | | Hemina | 0,01g |

**(1) Camargo (1964); (2) Rodrigues et al. (2006); (3) Roitman et al., 1977; a, hemina dissolvida em NaOH 0.1N; b, SFB (Soro Fetal Bovino), inativado à 56°C por 30 min e armazenado a -20°C.**

## 3.5 Caracterização morfológica por microscopia de luz, trasmissão e varredura

Para o estudo morfológico os tripanossomatídeos foram cultivados em meio de cultura LIT suplementado com 10% de soro bovino fetal, incubados a 28°C. Para a análise de microscopia óptica, os parasitas foram lavados com PBS 1X, fixados em lâminas com metanol e corados com Giemsa. Para análise de microscopia eletrônica de transmissão e varredura, os tripanossomatídeos foram lavados com PBS 1X, fixados com glutaraldeído (2,5%) suplementado com paraformaldeído (1%) em tampão de cacodilato de sódio (0,1M e pH 7,3) por 2 horas a temperatura ambiente e posteriormente fixados em um tampão de tretróxido de ósmio por 1 hora. Após a fixação, foi feita uma coloração com acetato de uranila por 16-18h, seguido de desidratação em etanol e inclusão em resina Spurr, como previamente descrito (Ventura et al., 2000). Os cortes ultrafinos foram corados com acetato de uranila e citrato de chumbo e examinados com um microscópio eletrônico Philips CM200.

### 3.6 Obtenção de DNA genômico dos tripanossomatídeos

Os tripanossomas obtidos em cultura foram lavados 2 vezes com PBS 1X, centrifugados por 10 min a 8.000g, e os "pellets" obtidos foram estocados a -20°C ou ressuspensos (1,0ml/$10^9$ tripanossomtídeos) em SE e mantidos em banho de gelo para extração de DNA. Após a adição de 0,5% de Sarkosil, 100 µg/ml de Pronase e 10 µg/ml de RNAse, o material foi incubado em banho-maria, a 55-60°C, por 1 a 2 h. Após esta incubação, a mistura foi extraída uma vez com Fenol:Tris 1:1, duas vezes com Fenol:$CHCl_3$ 1:1, duas vezes com $CHCl_3$: isoamílico 24:1 e uma vez com $CHCl_3$. Após a última extração, o DNA foi precipitado com acetato de sódio a 0,3M (pH 7,0) e 2 volumes de etanol 100% gelado, por incubação, por 12 a 15 horas, a –20°C. O DNA precipitado foi lavado com etanol a 70%; os "pellets" foram secos a 37°C e ressuspensos em TE. As soluções utilizadas estão descritas na Tabela 3.

Amostras diretas de tubos digestivos preservadas em etanol foram processadas utilizando o com o kit Wizard DNA Clean-Up System (Promega). As amostras de DNA de cultura e de tubos digestivos foram mantidas a 4°C e -20°C, respectivamente. Ambas foram quantificadas em espectrofotômetro.

**Tabela 3- Soluções utilizadas na obtenção de DNA genômico de tripanossomatídeos.**

| Soluções | Preparação | Volume | Recomendações |
|---|---|---|---|
| PBS 1X | NaCl (8.0g); KCl (0.2g); $Na_2HPO_4$ (1.15g); $KH_2PO_4$ (0.2g); em $H_2O$ bidestilada autoclavada. | 1000 mL | Autoclavar 120°C / 30 min. |
| SE | 99.5 ml NaCl (0.15M); 99.5 ml Tris (2.5mM) pH 8,0; 1ml EDTA (0.5M) pH 8,0. | 200 mL | Autoclavar 120°C / 30 min. |
| Sarkosil 10% | N-laurylsarcosine (1.0g) em $H_2O$ bidestilada autoclavada. | 10 mL | Fundir em banho-térmico 65°C. |
| Acetato de Sódio (3M) | $CH_3COONa$ 3H2O mw:136.08 (20.41g) em $H_2O$ bidestilada, acertar o pH para 7.0 com acido acético glacial diluído (1:10). | 50 mL | Autoclavar 120°C / 30 min. |
| TE (Tris-EDTA) | Tris-HCl 2M pH 7.5 (1.25ml); EDTA 0.5M pH 8.0 (0.5ml) em H2O bidestilada autoclavada. | 250 mL | Autoclavar 120°C / 30 min. |
| EDTA (0.5M) | EDTA (93.06g) em H2O bidestilada autoclavada, acertar o pH para 8.0 com NaOH (em lentilhas), aquecer para dissolver. | 500 mL | Autoclavar 120°C / 30 min. |

### 3.7 Análise de polimorfismo de tamanho e número de classes de moléculas de minicírculos de kDNA

Para a caracterização de moléculas de minicírculos de kDNA, cerca de 5μg de DNA genômico dos tripanossomatídeos foram desconcatenados por quebra mecânica em vortex, seguido de sucessivas passagens por ponteiras para micropipetas de 200μl. Após desconcatenadas, as moléculas de kDNA foram separadas por eletroforese em gel de agarose a 1%.

### 3.8 Amplificação de DNA por PCR ("Polymerase Chain Reaction")

Para as reações de PCR foi utilizada a seguinte mistura de reação: 100ng de DNA genômico; 100ng de cada "primer"; 200mM de cada dNTP; 5μl de tampão (200mM Tris-HCl, pH 8,4, 500mM KCl e 1,5 mM $MgCl_2$); 2,5 u de Taq DNA polimerase e água bidestilada deionizada e autoclavada (qsp 50μl). Os ciclos de amplificação e as temperaturas de anelamento foram definidos de acordo com os "primers" empregados (Tabela 4).

**Tabela 4. Ciclos de amplificação e temperaturas utilizadas nas diferentes reações de PCR**

| Gene e oligonucletídeos | Condições de amplificação |
|---|---|
| SSU rDNA (completo)<br>**KDR3 (5´GAT CTG GTT GAT TCT GCC AGT AG 3´)**<br>**KDR5 (5´GAT CCA GCT GCA GGT TCA CCT AC 3´)** | **1 ciclo: 3 min 94°C**<br>**29 ciclos: 1 min 94°C; 1 min 55°C; 1 min 72°C**<br>**1 ciclo: 10 min 72°C** |
| SSU rDNA (V7-V8)<br>**609F (5´ CAC CCG CGG TAA TTC CAG C 3´)**<br>**706R (5´ TTG AGG TTA CAG TCT CAG 3´)** | **1 ciclo: 3 min 94°C**<br>**29 ciclos: 1 min 94°C; 1 min 48°C; 1 min 72°C**<br>**1 ciclo: 10 min 72°C** |
| **oligonucleotídeos utilizados somente no sequenciamento:**<br>**1156F (5´CGT ACT GGT GCG TCA AGA GG 3´)**<br>**1156R (5´CCT CTG ACG CAC CAG TAC G 3´)**<br>**285F (5´GTG TTG ATT CAA TTC ATT C 3´)**<br>**285R (5´GAA TGA ATT GAA TCA ACA C 3´)**<br>**202F (5´ATG CTC CTC AAT GTT CTG 3´)**<br>**202R (5´CAG AAC ATT GAG GAG CAT3´)** | |
| gGAPDH<br>**GAP-tryF (5´GGN CGC ATG GTS TTY CAG G 3´)**<br>**GAP-tryR (5´CCC CAC TCG TTR TCR TAC C3´)** | **1 ciclo: 3 min 94°C**<br>**29 ciclos: 1 min 94°C; 1 min 55°C; 1 min 72°C**<br>**1 ciclo: 10 min 72°C** |
| **oligonucleotídeos utilizados somente no sequenciamento:**<br>**GAP 4R (5´GTG CTG GGG ATG ATG TTC3´)**<br>**GAP 3F (5´GTG AAG GCG CAG CGC AAC 3´)** | |
| ITS 1 rDNA<br>**T-ITSF (5´ GAT ATT TCT TCA ATA GAG GAA GC 3´)**<br>**5.8SR (5´ GGA AGC CAA GTC ATC CAT C 3´)** | **1 ciclo: 3 min 94 °C**<br>**29 ciclos: 1 min 94°C; 1 min 55 °C; 1 min 72°C**<br>**1 ciclo: 10 min 72°C** |
| **Genes do Endossimbionte**<br>16S rDNA (parcial) | |

| | |
|---|---|
| **680F (5'GTAGCAGTGAAATGCGTA3')**<br>**1486R (5'GATACGGCTACCTCCTTACG 3')** | **1 ciclo: 3 min 94°C**<br>**29 ciclos : 1 min 94°C; 1 min 60°C; 1 min 72°C**<br>**1 ciclo: 10 min 72°C** |
| ITS rDNA<br>**p1398F(5'ACACCATGGGAGTGGGTT3')**<br>**p23sRev (5'TCCAAGGCATCCACCGTA3')** | **1 ciclo: 3 min 94°C**<br>**34 ciclos: 1 min 94°C; 30 seg 51°C; 90 seg 72°C**<br>**1 ciclo: 10 min 72°C** |
| GAPDH<br>**GAPDH Endo F (5'AAG AGC TCA TTA TGA AGG TGG3')**<br>**GAPDH Endo R (5'TGG AAT CAT AYT CAT GGT TGC3')** | **1 ciclo: 3 min 94°C**<br>**34 ciclos: 1 min 94°C; 30 seg 50 °C; 90 seg 72°C**<br>**1 ciclo: 10 min 72°C** |
| Ribose 5-phosphate isomerase A<br>**Ribose 5F (5'GGR GTW GGW ACT GGA TCT AC3')**<br>**Ribose 5R (5' ACC ACA AGT WAY AAC ACC TGG3')** | **1 ciclo: 3 min 94°C**<br>**34 ciclos: 1 min 94°C; 30 seg 50 °C; 90 seg 72°C**<br>**1 ciclo: 10 min 72°C** |

### 3.9 Eletroforese de DNA em gel de agarose

As amostras de DNA genômico e fragmentos de DNA amplificado por PCR foram acrescidas de tampão de amostra (50% de glicerol; 0,4% de azul de bromofenol; 0,4% de xilenocianol) e submetidas à eletroforese em gel de agarose (1,5 a 2%) em tampão TAE (40mM de Tris-acetato; 2mM de EDTA, pH 8,0.) a 50 V/100 mA. Como marcador foi utilizado DNA Leader Mix (MBI Fermentas). Após a eletroforese, os géis foram corados com brometo de etídeo (0,5 ug/ml) ou GelRed® Nucleic Acid Gel Stain (Biotium) e fotografados em fotodocumentador ImageQuant 350 (GE Healthcare).

### 3.10 Purificação dos fragmentos de DNA amplificados por PCR

Fragmentos de DNA amplificados por PCR foram separados por eletroforese em gel de agarose a 1,5% e corados com brometo de etídeo ou GelRedR®Nucleic Acid Gel Stain (Biotium). Os fragmentos foram cortados dos géis e os DNAs foram extraídos da agarose em coluna Spin X (Costar®). Os produtos purificados foram clonados ou submetidos diretamente à reação de sequência.

### 3.11 Clonagem dos produtos amplificados por PCR

Fragmentos de DNA amplificados por PCR e purificados foram clonados em vetor pCR 2.1, utilizando os Kit TA Cloning (Invitrogen) e utilizados para a transformação em células dH10b. Os clones positivos foram ampliados por cultivo

em meio LB contendo 100µg/ml de ampicilina e purificados utilizando o sistema "Wizard Plus SV Minipreps DNA purification System" (Promega).

### 3.12 Sequenciamento de nucleotídeos

Fragmentos de DNA amplificados por PCR, clonados ou não, e purificados foram submetidos a reações de sequenciamento utilizando o kit Big Dye Terminator (Perkin Elmer), de acordo com especificações do fabricante, em sequenciador automático ABI PRISM 3500 Genetic Analyzer (Perkin Elmer) e ABI PRISM 3100 Genetic Analizer (Perkin Elmer). As reações foram submetidas a 30 ciclos: 15s. a 96$^{o}$C; 15s. a 50$^{o}$C; 4min. a 60$^{o}$C, com um ciclo inicial de 1min. a 96$^{o}$C.

Nas reações de sequenciamento foram utilizados os oligonucleotídeos citados na tabela 4. Para sequenciamento de fragmentos de DNA clonados em vetor pCR 2.1 (Kit TA Cloning – Invitrogen) foram utilizados os oligonucleotídeos M13F e M13R.

### 3.13 Alinhamento de sequências de nucleotídeos

Os cromatogramas das sequências foram analisados no programa Seqman do pacote de programas DNAstar (Nicholas et al., 1997). As sequências de nucleotídeos determinadas e as obtidas no GeneBank (http://www.ncbi.nlm.nih.gov/), foram alinhadas através do programa Clustal X (Thompson et al., 1997) alterando os parâmetros relativos à inserção de "indels" (peso de inserção=1, extensão=1). As sequências de aminoácidos e o alinhamento de nucleotídeos foram manualmente ajustadas no programa GeneDoc v. 2.7.000 (Nicholas et al., 1997).

### 3.14 Construção da matriz de similaridade

A partir do alinhamento das sequências de nucleotídeos e/ou aminoácidos dos genes em estudo, foi determinada uma matriz de similaridade (baseadas em distância *p* não corrigida) utilizando-se o programa Point Replacer v.2.2.

disponibilizado pelo autor (Alves, J. M.) no endereço http://reocities.com/CapeCanaveral/lab/3891/software.html.

### 3.15 Análises filogenéticas

Inferências filogenéticas foram determinadas pelos métodos de máxima parcimônia (MP), máxima verosimilhança (MV, "maximum likelihood") e análise bayesiana (B). As árvores de MP foram construídas utilizando o programa PAUP* v. 4.0b10 (Swofford, 1998) via busca heurística com 100 replicatas de adição aleatória dos terminais seguida de troca de ramos ("RAS-TBR Branch-breaking"). As análises de suporte por "bootstrap" foram feitas em 100 replicatas com os mesmos parâmetros empregados na busca.

As análises de MV foram realizadas no programa RAxML v7.0.4 (Stamatakis, 2006). Foram empregadas 500 replicatas usando GTR como modelo de substituição e 4 categorias de gama e diagramas obtidos por parcimônia como árvores iniciais. Os parâmetros do modelo de substituição empregado foram estimados durante a busca. O suporte de ramos foi estimado com 500 replicatas de "bootstrap".

As análises bayesianas foram executadas no programa MrBayes v.3.1.2 (Ronquist e Huelsenbeck, 2003). Foram empregadas 500.000 gerações usando GTR como modelo de substituição e 4 categorias de gama mais proporção de sítios invariantes. Para a construção do dendrograma final foram utilizados apenas os diagramas obtidos nas últimas 75 replicatas. Para a verificação de suporte de ramos nas análises bayesianas foram utilizados os valores de probabilidade a posteriori.

## 4. RESULTADOS E DISCUSSÃO

Os resultados obtidos durante o trabalho desenvolvido para essa tese estão resumidamente apresentados e discutidos abaixo. Optamos por apresentar apenas resultados já publicados, ou em fase de publicação, que constituem os artigos e manuscritos anexados no final da tese e listados abaixo.

### 4.1 Diversidade e filogenia de tripanossomatídeos parasitas de dípteros, coletados na América do Sul e África

**Anexo 1**

Phylogenetic Validation of the Genera *Angomonas* and *Strigomonas* of Trypanosomatids Harboring Bacterial Endosymbionts with the Description of New Species of Trypanosomatids and of Proteobacterial Symbionts

**Anexo 2**

Insect parasites of the genus *Angomonas*: Phylogeographical evidence of cryptic diversity, parasite-symbiont co-divergence and dispersion by blowflies throughout the Neotropics and Afrotropics

**Anexo 3**

Molecular Phylogenetic Redefinition of *Herpetomonas* (Kinetoplastea, Trypanosomatidae), a Genus of Insect Parasites Associated with Flies

Resumo dos trabalhos anexados

### 4.1.1 Validação filogenética gêneros *Angomonas* e *Strigomonas* de tripanossomatídeos que albergam endossimbiontes com descrição de duas novas espécies de tripanossomatídeos e simbiontes

**Anexo 1**: Phylogenetic Validation of the Genera *Angomonas* and *Strigomonas* of Trypanosomatids Harboring Bacterial Endosymbionts with the Description of New Species of Trypanosomatids and of Proteobacterial Symbionts

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Dentre os tripanossomatídeos monoxênicos de insetos, estão descritas cinco espécies que albergam endossimbiontes bacterianos: *Blastocrithidia culicis* (Chang 1975), *Crithidia oncopelti* (Gill and Vogel 1963), *Crithidia deanei* (Carvalho 1973), *Crithidia desouzai* e *Herpetomonas roitmani* (Faria e Silva et al. 1991, Fiorini et al. 1989, Motta et al. 1991). Neste trabalho os tripanossomatídeos que albergam endossimbiontes serão denominados SHTs (“Symbiont harboring trypanosomatids”, sigla em inglês). Os endossimbiontes de tripanossomatídeos são Betaproteobactérias intracitoplasmáticas obrigatórias, transmitidas verticalmente e serão denominados TPEs (“Trypanosomatid Proteobacterial Endosymbionts”). Geralmente há apenas um endossimbionte por célula o qual se divide sincronicamente com o tripanossomatídeo hospedeiro. Estudos sobre requerimentos nutricionais demonstraram que os simbiontes contribuem ativamente para o metabolismo dos tripanossomatídeos hospedeiros fornecendo vários nutrientes essenciais. O posicionamento taxonômico dos tripanossomatídeos portadores de simbionte tem sido controverso, pois os flagelados com e sem simbiontes dos gêneros *Blastocrithidia*, *Herpetomonas* e *Crithidia* diferem em muitas características morfológicas, bioquímicas e moleculares. Além disso, a morfologia coanomastigota nos tripanossomatídeos portadores de simbionte também não é ortodoxa. Assim, com base na localização do cinetoplasto posteriormente ao núcleo (forma designada no presente estudo de opistomorfa) Souza e Corte-Real (1991) propuseram a

classificação de *C. deanei* em um novo gênero, chamado *Angomonas*. Posteriormente, com base na morfologia e nas características do kDNA Brandão et al. (2000) restabeleceram o gênero *Strigomonas* para acomodar *C. oncopelti* e propuseram a classificação de *C. deanei* e *C. desouzai* no gênero *Angomonas*. Entretanto essa classificação não foi adotada, provavelmente devido à falta de dados taxonômicos suportados por análises filogenéticas moleculares. Para avaliar a proposição dos gêneros *Angomonas* e *Strigomonas* partindo do conhecimento mais detalhado das relações filogenéticas e taxonômicas dos tripanossomatídeos que albergam endossimbiontes caracterizamos, no presente estudo, todas as espécies de SHTs disponíveis, bem como novos isolados identificados segundo critérios nutricionais, morfológicos e moleculares a partir do exame de mais de 500 culturas de tripanossomatídeos estabelecidas. O sequenciamento da região V7V8 da subunidade menor do RNA (SSU rRNA) distinguiu seis genótipos principais entre os 13 isolados examinados, divididos em dois subclados correspondentes aos gêneros *Angomonas* e *Strigomonas* anteriormente propostos. Árvores filogenéticas geradas com as sequências completas dos genes SSU rRNA e e gGAPDH ("glycosomal Glyceraldehyde Phosphate Dehydrogenase") agruparam as espécies que albergam simbiontes em um único clado monofilético e altamente suportado, independentemente do gene ou do alinhamento utilizado. Essas análises posicionaram as espécies sem simbiontes dos gêneros *Crithidia*, *Herpetomonas* e *Blastocrithidia* fora do clado dos tripanossomatídeos com simbionte e em ramos separados. A separação do clado dos SHTs em dois subclados, um deles correspondendo ao gênero *Angomonas* e o outro ao gênero *Strigomonas* (que inclui *Blastocrithidia culicis*) foi fortemente suportada com ~ 13% e ~12% de divergência de SSU rRNA e gGAPDH respectivamente. O gênero *Strigomonas* foi composto por três espécies: *S. culicis* comb. nov., *S. oncopelti* comb. nov and *S. galati* sp.nov. O gênero *Angomonas* foi dividido em dois subgrupos: deanei e desouzai. O subgrupo deanei é composto por *A. deanei* comb. nov, e mais seis parátipos, incluindo *H. roitmani,* espécie anteriormente classificada no gênero *Herpetomonas*. O subgrupo desouzai compreende *A. desouzai* comb. nov, dois parátipos e uma nova espécie denominada *Angomonas ambiguus* sp. nov. Sequências do espaçador interno transcrito ITS rDNA e polimorfismo de tamanho de minicírculos do kDNA revelaram grande heterogeneidade genética dentro dos gêneros *Angomonas* e *Strigomonas*.

Inferências filogenéticas baseadas em sequências do 16S rDNA e ITS rDNA dos simbiontes posicionaram todos os endossimbiontes em um clado monofilético dentro de Betaproteobacteria. As análises também revelaram a separação dos simbiontes em dois grupos distintos, correspondentes aos hospedeiros *Strigomonas* e *Angomonas,* e permitiram a classificação de todos os TPEs como "Candidatus" (como recomendado por Murray e Stackebrandt, 1995) pertencentes ao gênero Kinetoplastibacterium (Du et al., 1994a). De acordo com as divergências de sequências do 16S rRNA, propomos a classificação dos simbiontes de tripanossomatídeos em cinco espécies distintas, mantendo a classificação original de "*Candidatus* Kinetoplastibacterium crithidii" e "*Candidatus* K. blastocrithidii" para os simbiontes de *A. deanei* e *S. culicis* respectivamente, e denominando os simbiontes de *S. oncopelti*, *S. galati* e *A. desouzai* respectivamente de "*Ca*. K. oncopeltii", "*Ca*. K. galatii" e "*Ca*. K. desouzaii". Embora não se conheça o tempo e nem os mecanismos que originaram essa associação simbiótica, hospedeiros e simbiontes partilham uma história evolutiva comum e antiga, corroborada no presente estudo comparando as filogenias de tripanossomatídeos de SHTs e a de seus respectivos endossimbiontes. Os resultados revelaram uma congruência global entre as filogenias do hospedeiro e do simbionte, com paridade completa em nível de gênero e uma exceção ao nível de espécie: *A. ambiguus*, é mais relacionada com *A. desouzai*, cujo simbionte, entretanto, é filogeneticamente mais próximo do simbionte de *A. deanei*. Considerando que provavelmente todos os TPEs tiveram uma origem comum, estes resultados indicam que os simbiontes e seus hospedeiros tripanossomatídeos provavelmente estejam evoluindo em taxas evolutivas diferentes. A maioria dos SHTs aqui caracterizados pertence ao gênero *Angomonas* e, com exceção de *A. deanei* (espécie isolada de hemíptero predador), foram isolados de Dipteros do Brasil, o que permite sugerir os Dipteros como os hospedeiros mais susceptíveis a infecções por *Angomonas* sp.

### 4.1.2 Parasitas de insetos do gênero Angomonas: evidência filogeográfica da diversidade, da co-divergência parasita-simbionte e dispersão por varejeiras da região Neotropical e Afrotropical

**Anexo 2.** Insect parasites of the genus *Angomonas*: Phylogeographical evidence of cryptic diversity, parasite-symbiont co-divergence and dispersion by blowflies throughout the Neotropics and Afrotropics

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A família Calliphoridae formada por moscas de reflexos metálicos e conhecidas como “moscas varejeiras” ou “blowfly”, é um grupo diverso, heterogêneo e cosmopolita, do qual fazem parte espécies de importância médica, veterinária e forense. No Novo Mundo, a família Calliphoridae compreende espécies nativas por exemplo, do gênero *Cochliomyia* e espécies cosmopolitas, como as dos gêneros *Chrysomya* e *Lucilia,* originárias do Velho Mundo. Em estudos prévios evidenciamos a associação de tripanossomatídeos dos gêneros *Herpetomonas* e *Angomonas* com moscas varejeiras e sugerimos as Calliphoridae como hospedeiras preferenciais de *Herpetomonas* e *Angomonas*. Contudo, a associação *Angomonas* / Calliphoridae foi pouco estudada até o momento, dada a insuficiência de dados biogeográficos e moleculares para um estudo filogeográfico mais abrangente. A falta de informação dificulta compreender como as moscas varejeiras nativas e exóticas contribuíram para a dispersão de tripanossomatídeos do gênero *Angomonas* na região neotropical. Neste estudo, avaliamos a biogeografia e a diversidade genética dos tripanossomatídeos portadores de simbiontes do gênero *Angomonas* que parasitam moscas varejeiras. Comparamos também a composição de espécies de tripanossomatídeos encontradas nos dípteros presentes nas regiões Neotropical e Afrotropical. O sequenciamento da região V7V8 da subunidade menor do RNA (SSU rRNA) (barcoding) de 364 culturas de tripanossomatídeos isolados de dípteros coletados na América do Sul e na África identificou 70 culturas (20%) como do gênero *Angomonas;* destas, 50 (71%) foram isoladas de moscas varejeiras. Além de culturas, 13 isolados de *Angomonas* foram identificados por PCR a partir dos tubos digestivos. Deste modo, 83 isolados pertencentes ao gênero *Angomonas* foram encontrados neste estudo, sendo 61 de *A. deanei*, 6 de *A. ambiguu*s e 16 de *A. desouzai.* As três espécies foram encontradas tanto na América do Sul quanto na

África, principalmente em dípteros dos gêneros *Chrysomya* e *Lucilia* (61; 73%), seguido por moscas da família Muscidae (12; 14%). Análises filogenéticas baseadas em sequências do gene gGAPDH de *Angomonas,* procedentes tanto de culturas quanto de tubos digestivos de diferentes hospedeiros e regiões geográficas corroboraram a monofilia do gênero *Angomonas* e sua divisão em três clados principais formados por *A. deanei*, *A. ambiguus* e *A. desouzai*. As análises também revelaram uma diversidade intra-específica de *A. deanei* com a formação de quatro subclados (genótipos Ade1-Ade4) e também de *A. desouzai* cujos isolados distribuíram-se em dois subclados (genótipos Ads1-Ads2). Os isolados de *A. ambiguus* mostraram-se mais homogêneos. As filogenias dos endossimbiontes baseadas em sequências polimórficas de GAPDH, Rpi (ribose 5-fosfato isomerase A) e ITS rDNA foram congruentes com as filogenias dos respectivos tripanossomatídeos hospedeiros. Os quatro subclados de *A. deanei* também foram formados nas filogenias do simbionte (genótipos SAde1-SAde4) assim como os dois subclados de *A. desouzai* (SAds1-SAds2). Corroborando dados anteriores, o simbionte de *A. ambiguus* foi filogeneticamente mais próximo do simbionte de *A. deanei,* posicionando-se no subclado SAde3. As três espécies de *Angomonas* foram encontradas em dípteros da América do Sul e África, principalmente em moscas varejeiras dos gêneros *Chrysomya* e *Lucilia*. O gênero *Chrysomya* era restrito ao Velho Mundo até meados da década de setenta quando foram relatadas três espécies no Brasil. Estes resultados permitem supor que varejeiras do Velho Mundo foram responsáveis pela dispersão do gênero *Angomonas* no neotrópico. Apesar da forte associação de *Angomonas* com Calliphoridae, essas moscas também podem abrigar tripanossomatídeos de outros gêneros, enquanto moscas de outras famílias também podem conter *Angomonas*. Este é o estudo mais abrangente sobre tripanossomatídeos que parasitam varejeiras e de espécies de *Angomonas*. A análise da associação entre tripanossomatídeos, seus respectivos simbiontes e Calliphoridae permite sugerir que a dispersão das moscas varejeiras contribuiu para a diversidade e distribuição do gênero *Angomonas*.

### 4.1.3 Redefinição filogenética molecular de *Herpetomonas* (Kinetoplastea, Trypanosomatidae), um gênero parasitas de insetos associados com moscas

**Anexo 3.** Molecular Phylogenetic Redefinition of *Herpetomonas* (Kinetoplastea, Trypanosomatidae), a Genus of Insect Parasites Associated with Flies

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O gênero *Herpetomonas,* composto por tripanossomatídeos monoxênicos de insetos, foi criado para acomodar flagelados que apresentam em seus ciclos de vida formas promastigotas e opistomastigotas. Nas formas promastigotas, o cinetoplasto localiza-se anteriormente ao núcleo, enquanto que nas opistomastigotas o cinetoplasto situa-se posteriormente ao núcleo. Em princípio, a criação do gênero para acomodar flagelados de formas opistomastigotas presentes nos insetos e em culturas, deveria facilitar a identificação de novos tripanossomatídeos no gênero. No entanto, opistomastigotas podem ser raras e até mesmo ausentes em de insetos e em culturas, podendo, além disso, ser confundidas com as formas opistomorfas, encontradas nos gêneros *Angomonas* e *Strigomonas*. Estas incertezas geraram a classificação equivocada de espécies de *Herpetomonas* em gêneros que também exibem formas promastigotas (*Leptomonas, Phytomonas*) e, inversamente, a classificação como *Herpetomonas* de tripanossomatídeos agora reclassificados em gêneros diferentes. Neste estudo, posicionamos um grande número de *Herpetomonas* spp. na árvore filogenética da família Trypanosomatidae de modo a inferir as relações entre as espécies do gênero. Com o objetivo de rever a taxonomia do gênero *Herpetomonas* baseada em análises filogenéticas e parâmetros morfológicos, sequenciamos a região V7V8 da subunidade menor do gene ribossômico (SSU rRNA) de 527 tripanossomatídeos isolados de insetos. Cinquenta e dois flagelados foram relacionados com espécies conhecidas de *Herpetomonas*, 90% dos quais foram isolados de Diptera. O dendograma resultante da análise do relacionamento genético entre as espécies de referência e os novos isolados revelou a distribuição dos flagelados em 11 clados distintos. Para o posicionamento filogenético de *Herpetomonas* dentro da família Trypanosomatidae, sequenciamos

os genes gGAPDH e SSU rRNA. Nas árvores filogenéticas, os flagelados selecionados agruparam-se em um único ramo monofilético fortemente suportado. Com base nas distâncias genéticas das sequências dos genes gGAPDH e SSU rRNA juntamente com as análises filogenéticas distinguimos 13 espécies de *Herpetomonas*: seis espécies já conhecidas (*H. muscarum / H. megaseliae*, *H. samuelpessoai*, *H. ztiplika*, *H. trimorpha* e *H. mariadeanei)*; cinco novas espécies (*H. isaaci* n. sp., *H. puellarum* n. sp., *H. elegans* n. sp., *H. modestus* n. sp. e *H. wanderleyi* n. sp.); e dois isolados, anteriormente classificados como *Leptomonas* spp., que foram reclassificados neste trabalho como *Herpetomonas*: *H. samueli* n. comb. e *H. costoris* n. comb. Dentro da família Trypanosomatidae, devido à sua alta divergência genética em relação ao SSU rRNA e gGAPDH, sequências ITSrDNA têm sido frequentemente utilizadas para inferências filogenéticas entre espécies proximamente relacionadas. Neste trabalho foram analisadas estruturas primárias e secundárias das 11 espécies de *Herpetomonas.* As Sequências de ITS1 rDNA foram altamente variáveis quanto ao comprimento, variando de 145 a 770 pb. A análise das estruturas primária e secundária do ITS1 rDNA corroborou os resultados obtidos com os genes SSU rRNA e gGAPDH. Esta é a primeira vez que a comparação de estruturas secundárias foi utilizada para fins filogenéticos e taxonômicos em tripanossomatídeos. Análises morfológicas baseadas em microscopia de luz e eletrônica de culturas de *Herpetomonas* na fase logarítmica de crescimento revelou grande polimorfismo inter e intra-específico, tanto no comprimento quanto no formato do corpo dos flagelados. Este estudo revelou que a diversidade morfológica e molecular encontrada em *Herpetomonas* é a maior quando comparada à de todos os outros gêneros filogeneticamente validados de tripanossomatídeos monoxênicos de insetos. Nossos resultados também mostraram que algumas espécies de *Herpetomonas* são parasitas generalistas de moscas, tão cosmopolitas quanto seus hospedeiros, de modo que a origem geográfica e hospedeiro de origem mostram-se insuficientes como critérios taxonômicos, não devendo, portanto, ser usados sozinhos para a identificação de gêneros e espécies de tripanossomatídeos de insetos. É cada vez mais evidente que filogenias moleculares permitem uma completa reavaliação da taxomonia dos tripanossomatídeos de insetos. Nesse sentido, a proposta de dissociar o gênero *Herpetomonas* de sua clássica definição morfológica inicial é um importante passo nessa direção. Análises filogenéticas

baseadas em sequências de SSU rRNA e gGAPDH demonstraram que *Herpetomonas* é um gênero monofilético constituído por espécies solitárias juntamente com espécies reunindo isolados de diversos hospedeiros e origens geográficas.

## 5. CONCLUSÃO

- O estudo de tripanossomatídeos parasitas de moscas capturadas na América do Sul (Brasil, Venezuela e Colômbia) e África (Moçambique, Guiné Bissau e Madagascar) revelou que os gêneros *Angomonas* e *Herpetomonas* apresentam forte associação com moscas.
- Análises filogenéticas baseadas nos genes SSU rRNA e gGAPDH apoiaram a monofilia de todos os tripanossomatídeos com endossimbiontes, a divisão em dois subclados correspondentes aos gêneros *Strigomonas* e *Angomonas* e a descrição de uma nova espécie de cada gênero.
- A comparação *(DNA barcoding*) de 364 culturas de moscas coletadas na América do Sul e África revelou que 20% são do gênero *Angomonas*, 71% delas de Calliphoridae. Análises de amostras de tubo digestivo detectaram *Angomonas* em 51% das moscas. De 83 tripanossomatídeos de moscas, 61 foram identificados como *A. deanei*, 16 como *A. desouzai* e 6 como *A. ambiguu*s. *A. deanei* e *A. desouzai* apresentaram diversos genótipos.
- Os principais hospedeiros de *Angomonas* são moscas dos gêneros *Chrysomya* e *Lucilia* (51%) sugerindo que a dispersão recente dessas moscas deve ter tido um papel importante na distribuição mundial das mesmas espécies e genótipos de *Angomonas*.
- Análises filogenéticas revelaram três novas espécies de endossimbiontes e congruência entre a maioria das espécies hospedeiras e seus simbiontes, sugerindo co-divergência.
- As filogenias inferidas para os tripanossomatídeos de moscas permitiram validar o gênero *Herpetomonas*, que agora compreende 13 espécies válidas, incluindo 5 novas espécies descritas com esse estudo.
- As espécies de *Angomonas* e *Herpetomonas* são parasitas generalistas de moscas, tão cosmopolitas quanto seus hospedeiros.

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**ORIGINAL PAPER**

# Phylogenetic Validation of the Genera *Angomonas* and *Strigomonas* of Trypanosomatids Harboring Bacterial Endosymbionts with the Description of New Species of Trypanosomatids and of Proteobacterial Symbionts

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**We comparatively examined the nutritional, molecular and optical and electron microscopical characteristics of reference species and new isolates of trypanosomatids harboring bacterial endosymbionts. Sequencing of the V7V8 region of the small subunit of the ribosomal RNA (SSU rRNA) gene distinguished six major genotypes among the 13 isolates examined. The entire sequences of the SSU rRNA and glycosomal glyceraldehyde phosphate dehydrogenase (gGAPDH) genes were obtained for phylogenetic analyses. In the resulting phylogenetic trees, the symbiont-harboring species clustered as a major clade comprising two subclades that corresponded to the proposed genera *Angomonas* and *Strigomonas*. The genus *Angomonas* comprised 10 flagellates including former *Crithidia deanei* and *C. desouzai* plus a new species. The genus *Strigomonas* included former *Crithidia oncopelti* and *Blastocrithidia culicis* plus a new species. Sequences from the internal transcribed spacer of ribosomal DNA (ITS rDNA) and size polymorphism of kinetoplast DNA (kDNA) minicircles revealed considerable genetic heterogeneity within the genera *Angomonas* and *Strigomonas*. Phylogenetic analyses based on 16S rDNA and ITS rDNA sequences demonstrated that all of the endosymbionts belonged to the Betaproteobacteria and revealed three new species. The congruence of the phylogenetic trees of trypanosomatids and their symbionts support a co-divergent host-symbiont evolutionary history.**


**Key words:** Trypanosomatids; phylogeny; evolution; Betaproteobacteria; endosymbiont; co-divergence.

## Introduction

The protistan family Trypanosomatidae (Euglenozoa, Kinetoplastea) comprises flagellates that are obligate parasites of all classes of vertebrates

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including man, plants and invertebrates, mainly insects. The parasites of plants (genus *Phytomonas*) and vertebrates (genera *Trypanosoma* and *Leishmania*) may be pathogenic in their hosts to which they are transmitted by invertebrate vectors. Trypanosomatid parasites exclusively of insects constitute the largest segment of the family and have been reported in more than 400 insect species worldwide. Their preferential hosts are dipterans and hemipterans, in which they are generally non-pathogenic, develop in the digestive tract, and are transmitted by coprophagy or predation (Camargo 1998; Hoare 1972; Vickerman 1994; Wallace 1966). The trypanosomatids of insects were reorganized by Hoare and Wallace (1966) in the genera *Crithidia, Leptomonas, Herpetomonas* and *Blastocrithidia*. Two genera, *Wallaceina* and *Sergeia,* were later added to the family (Merzlyak et al. 2001; Svobodová et al. 2007). Five species of insect trypanosomatids have been reported to carry cytoplasmic bacterial endosymbionts: *Blastocrithidia culicis* (Chang 1975), *Crithidia oncopelti* (Gill and Vogel 1963), *Crithidia deanei* (Carvalho 1973), *Crithidia desouzai* and *Herpetomonas roitmani* (Faria-e-Silva et al. 1991; Fiorini et al. 1989; Motta et al. 1991). Symbiont harboring trypanosomatids are hereafter denoted SHTs.

Endosymbionts of trypanosomatids are obligate intra-cytoplasmic Betaproteobacteria of vertical transmission and are hereafter denoted TPEs (trypanosomatid proteobacterial endosymbionts). Typically, there is one symbiont per cell, which implies that the symbiont and host cell divide synchronously (Du et al. 1994b; Motta et al. 2010). The endosymbionts actively contribute to the metabolism of the trypanosomatid host by providing essential nutrients (Chang and Trager 1974; Frossard et al. 2006; Kořený et al. 2009; Roitman and Camargo 1985). SHTs and trypanosomatid species that do not harbor symbionts differ with respect to their ability to synthesize essential amino acids (Alfieri and Camargo 1982; Camargo and Freymuller 1977). They also differ with respect to several ultrastructural features (Cavalcanti et al. 2008; Freymuller and Camargo 1981). SHTs that are artificially freed from their symbionts by chloramphenicol treatment (aposymbiotic strains) become fastidious and lose many of their biosynthetic capabilities (Roitman and Camargo 1985) and differ from the respective symbiotic strains in their surface glycoconjugates and proteolytic enzymes (d'Avila-Levy et al. 2001, 2008)

Species of *Crithidia* and *Herpetomonas*-harboring symbionts began to be studied about 80 years ago when trypanosomatids from insects and plants cultured in the USA were collectively named *Herpetomonas oncopelti* (Noguchi and Tilden 1926). One of the cultures was successively classified in the genus *Leptomonas,* subgenus/genus *Strigomonas* (Lwoff and Lwoff 1931; Thomson 1932) and was finally designated *Crithidia oncopelti* (Gill and Vogel 1963). This culture was the first trypanosomatid reported to carry bacteria-like symbionts.

The taxonomic positioning of SHTs has been controversial because *Blastocrithidia, Herpetomonas* and *Crithidia* with and without symbionts differed in several morphological, biochemical and molecular features. For instance, in the genus *Crithidia,* species harboring symbiont differ from non-harboring symbiont species in kDNA minicircle and ribosomal DNA restriction profiles, spliced leader gene repeat sequences and positioning in phylogenetic trees (Brandão et al. 2000; Camargo et al. 1982, 1992; Fernandes et al. 1997; Hollar et al. 1998; Maslov et al. 1996, 2010; Merzlyak et al. 2001; Teixeira et al. 1995). In addition, the morphology of SHT choanomastigotes also is unorthodox. Accordingly, Souza and Côrte-Real (1991) proposed that *C. deanei* be classified in a new genus, *Angomonas,* based on the morphology of choanomastigotes displaying the kinetoplast behind the nucleus. We designated these peculiar forms as opisthomorphs. Opisthomorphs differ from classical choanomastigotes in the positioning of the kinetoplast whereas opisthomorphs differ from conventional opisthomastigotes (typical of *Herpetomonas* species) in morphology and ability to multiply (Faria-e-Silva et al. 1996; Teixeira et al. 1997). Later on, Brandão et al. (2000) proposed the reinstatement of the genus *Strigomonas* to accommodate *C. oncopelti* and the placement of *C. deanei* and *C. desouzai* in *Angomonas* based on morphology and kDNA features. However, this proposition has not been adopted probably due to a lack of accompanying taxonomic data supported by molecular phylogenetic analyses.

In recent years, phylogenetic analysis has emerged as a powerful tool to dismiss or create new genera and species of Trypanosomatidae. Phylogenetic trees have revealed that all SHT species cluster together and are clearly separated from the other species of genera *Crithidia, Herpetomonas* and *Blastocrithidia,* in which they were traditionally classified. These results evidenced the polyphyly of these three genera (Hollar et al. 1998; Maslov et. al. 2010; Merzlyac et al. 2001; Svobodová et al. 2007; Yurchenko et al. 2008, 2009).

To evaluate the proposition of the genera *Angomonas* and *Strigomonas* based on a detailed understanding of the phylogeny and taxonomic relationships of SHTs, we characterized all the available SHT species and examined more than 500 cultures of trypanosomatids for new SHTs via nutritional and molecular screening. Barcoding of the variable V7V8 region of the SSU rRNA gene of 13 putative SHTs followed by SSU rRNA, gGAPDH and ITS1 rDNA phylogenetic analyses supported the division of SHTs into two highly consistent sister clades corresponding to the genera *Angomonas* and *Strigomonas.* In addition, these analyses enabled the description of one new species within each genus. Analyses of the 16S rRNA and ITS1 rDNA sequences of the endosymbionts revealed three new species of obligate symbiotic Betaproteobacteria and provided new information concerning the history of trypanosomatid-symbiont co-evolution.

## Results

### Isolation and Nutritional Selection of Trypanosomatids Harboring Bacterial Symbionts

In this study, among ~500 cultures examined, 13 trypanosomatids (Table 1) were confirmed to harbor symbionts according to nutritional, molecular and morphological analyses. Our laboratory routinely screens for SHTs based on their ability to grow in hemin-free culture medium. This medium was selected because symbionts provide hemin, an essential nutrient that is not synthesized by trypanosomatids without symbionts (Chang et al. 1975). All of the flagellates listed in Table 1 sustained growth on hemin-free medium for at least 10 successive passages in culture, excluding the laboratory-produced lineages that were freed of their symbionts (aposymbiotic lineages) by antibiotic treatment.

### Barcoding of Trypanosomatids by Means of V7V8 SSU rRNA Gene Sequencing

Initially we barcoded the variable V7V8 region of the SSU rRNA gene of all trypanosomatids found to be prototrophic for hemin. The flagellates examined could be separated into two groups, which corresponded to the proposed genera *Angomonas* and *Strigomonas* (Fig. 1B). *Strigomonas* included the former *Blastocrithidia culicis, C. oncopelti* and the new genotype TCC219 (Table 1, Fig. 1B). *Angomonas* included two subgroups: one comprises *C. deanei* (TCC036E) and *H. roitmani* (TCC080E) and the isolates TCC263E, 1445, 1743 and 1756, the other contains *C. desouzai* and the isolates TCC1279 and 1429 plus the new genotype TCC1780 (Table 1, Fig. 1B). The aposymbiotic strains of *C. deanei* (TCC044E) and *C. oncopelti* (TCC045E) shared sequences identical to those of the SHTs from which they derived. The entire SSU rRNA and gGAPDH genes of these flagellates were then sequenced to infer their phylogenetic positioning within the Trypanosomatidae tree.

### Phylogenetic Inferences Using Independent and Combined SSU rRNA and gGAPDH Genes

SSU rRNA and gGAPDH gene sequences of SHTs were aligned with the corresponding sequences of trypanosomatids representative of all other monoxenous (one host) genera and of the heteroxenous genera *Leishmania* and *Trypanosoma.* Phylogenetic analyses using independent or combined gene sequences generated highly concordant trees by parsimony (P), maximum likelihood (ML) and Bayesian inferences (BI) (Fig. 1A, B).

In all phylogenetic trees, SHT species clustered together in a monophyletic assemblage irrespectively of the gene, alignment, taxon coverage or analytical method used. The tree topologies followed the general pattern of Trypanosomatidae phylogenetic trees (Hamilton et al. 2007; Maslov et al. 2010; Yurchenko et al. 2009). Large genetic distances in the SSU rRNA and gGAPDH genes separated the SHT clade from the other trypanosomatid clades. Species of *Crithidia, Herpetomonas* and *Blastocrithidia* that do not harbor symbionts were positioned distantly from the SHT clade in separated branches (Fig. 1A).

Phylogenetic analyses strongly supported the partition of the SHT clade into two subclades corresponding to the genera *Angomonas* and *Strigomonas* (including *Blastocrithidia culicis*). These subclades are separated by large sequence divergences in SSU rRNA (~13%) and gGAPDH (~12%) genes. The genus *Strigomonas* is represented by three species: *S. culicis* comb. nov., *S. oncopelti* comb. nov and *S. galati* sp. nov, which correspond to the new genotype TCC219. Sequences of SSU rRNA and gGAPDH genes diverged respectively ~7.0% and ~8.0% between *S. culicis* and *S. oncopelti*, ~8.0% and ~6.0% between *S. galati* and *S. oncopelti*, and ~8.0% and ~7.0% between *S. culicis* and *S. galati* (Table 2).

**Table 1.** Symbiont harboring trypanosomatids, host and geographic origin and gene sequences from hosts and symbionts.

| Organism | Host origin | | | Geographical origin | GenBank acession number | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | Host gene sequences | | | Symbiont gene sequences | |
| | order | family | species | City/State/country | SSU rRNA | ITS1 rDNA | gGAPDH | 16S rDNA | ITS rDNA |
| *Strigomonas culicis* | | | | | | | | | |
| TCC012E | D | Cul. | *Aedes vexans* | USA | HM593009 | HQ594516/17 | EU079137 | L29265 | L29265 |
| *Strigomonas oncopelti* | | | | | | | | | |
| TCC043E | H | Lyg. | *Oncopeltus sp* (?) | USA | AF038025 | HM593041/42 | EU079134 | HM592997 | HM593045 |
| TCC045E (apo) | H | Lyg. | *Oncopeltus sp* (?) | USA | – | – | – | – | – |
| *Strigomonas galati* | | | | | | | | | |
| TCC219 | D | Phl. | *Lutzomya almerioi* | Bodoquena/MS/BR | HM593010 | HM593025/26 | HM593018 | HM592998 | HM593046 |
| *Angomonas deanei* | | | | | | | | | |
| TCC036E | H | Red. | *Zelus leucogrammus* | Goiânia/GO/BR | HM593011 | HM593033/34 | HM593022 | HM593003 | HM593050 |
| TCC044E (apo) | H | Red. | *Zelus leucogrammus* | Goiânia/GO/BR | – | – | – | – | – |
| TCC080E | D | Syr. | *Ornidia obesa* | Alfenas/MG/BR | HM593012 | – | EU079130 | HM593004 | – |
| TCC263E | D | Cal. | *Chrysomya putoria* | Rolândia/PR/BR | AF038024 | – | EU079131 | HM593008 | – |
| TCC1445 | D | Cal. | *Chrysomya megacephala* | Campo Grande/MS/BR | HM593013 | HM593035/36 | HM593023 | HM593005 | HM593051 |
| TCC1743 | D | Cal. | *Lucilia cuprina* | São Paulo/SP/BR | HM593043 | HM593037/38 | – | HM593006 | HM593052 |
| TCC1756 | D | Cal. | *Lucilia cuprina* | Monte Negro/RO/BR | HM593014 | HM593039/40 | HM593024 | HM593007 | HM593053 |
| *Angomonas desouzai* | | | | | | | | | |
| TCC079E | D | Syr. | *Ornidia obesa* | Alfenas/MG/BR | HM593016 | HM593027/28 | HM593020 | HM592999 | HM593047 |
| TCC1279 | D | Cal. | *Lucilia eximia* | Campo Grande/MS/BR | HM593017 | HM593029/30 | HM593021 | HM593000 | HM593048 |
| TCC1429 | D | Cal. | *Lucilia eximia* | Campo Grande/MS/BR | HM593044 | – | – | HM593001 | – |
| *Angomonas ambiguus* | | | | | | | | | |
| TCC1780 | D | Cal. | *Chrysomya albiceps* | Campo Grande/MS/BR | HM593015 | HM593031/32 | HM593019 | HM593002 | HM593049 |

Orders: H, Hemiptera; D, Diptera
Families: Lyg, Lygaeidae; Phl, Phlebotomidae; Red. Reduviidae; Cal., Calliphoridae, Syr., Syrphidae
Apo, aposymbiotic strains

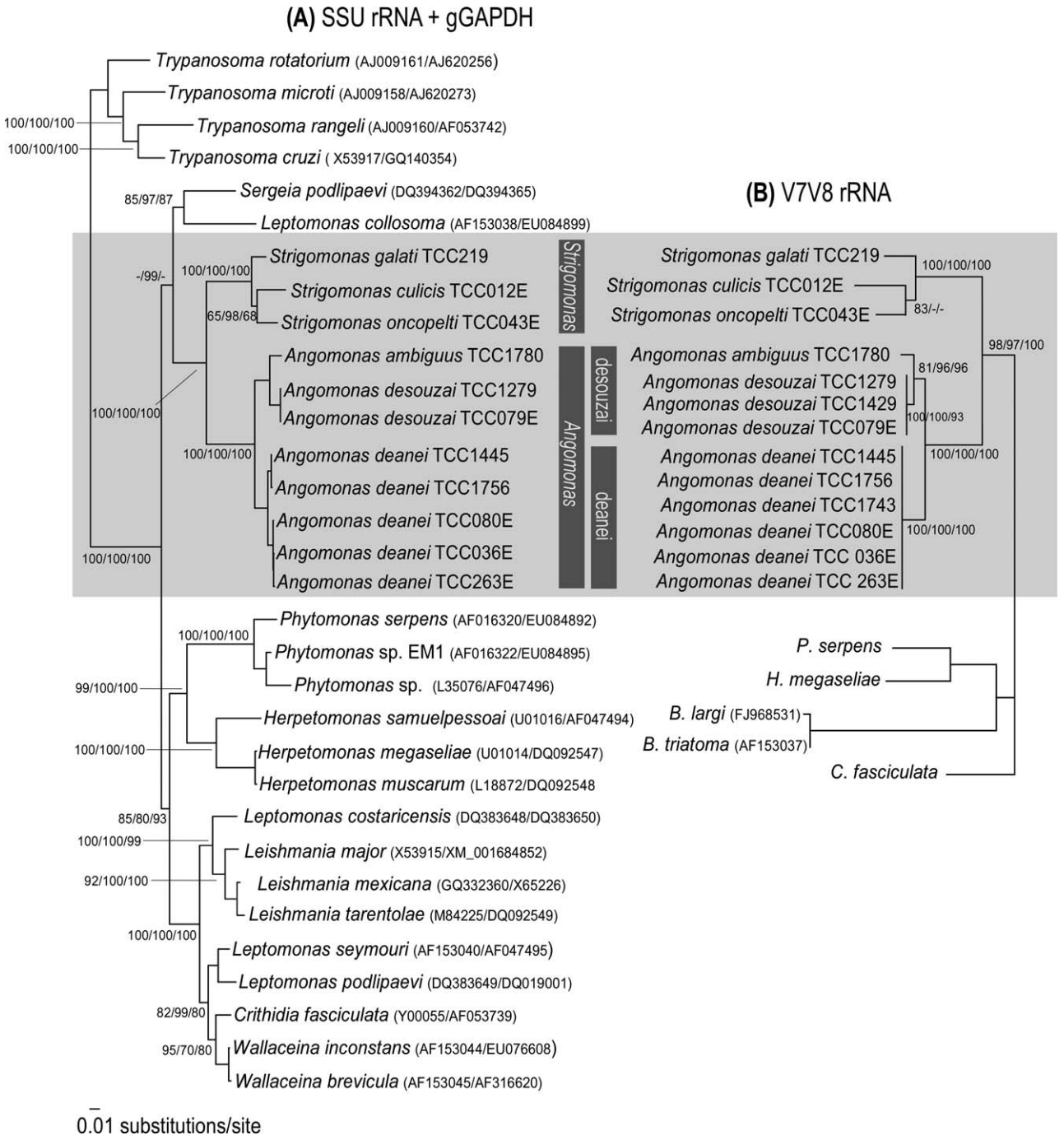


**Figure 1. (A)** Phylogenetic tree of the symbiont harboring trypanosomatids (SHTs) and representatives of distinct trypanosomatid genera inferred by maximum likelihood (ML) analysis based on combined SSU rRNA and gGAPDH gene sequences (alignment A4, 2782 characters, Ln=-15517.691576). **(B)** ML analysis of the SHT clade using sequences from the variable V7-V8 region of SSU rRNA (alignment A1, 1018 characters, Ln=-3196.995872). The accession numbers of SSU rRNA and gGAPDH sequences in the Genbank are in the Table 1 for SHT and within parenthesis for the remaining organisms. Multiple values of at major nodes are in order ML, BI and P support values derived from 1000 (A) or 500 (B) replicates. The species of the genera *Strigomonas* and *Angomonas* (subgroups deanei and desouzai) were all nested in the SHT clade shown in the shadowed box.

**Table 2.** Nucleotide divergences of SSU rRNA, gGAPDH and ITS rDNA sequences between trypanosomatid host species of the genera *Strigomonas* and *Angomonas*, and between 16S rDNA and ITS rDNA sequences of their respective endosymbionts.

| Trypanosomatid host | *S. culicis* | *S. oncopelti* | *S. galati* | *A. deanei* | *A. desouzai* | *A. ambiguus* |
|---|---|---|---|---|---|---|
| SSU rRNA | | | | | | |
| *S. culicis* | – | | | | | |
| *S. oncopelti* | 7.0% | – | | | | |
| *S. galati* | 8.0% | 8.0% | – | | | |
| *A. deanei* | 13% | 13% | 12% | – | | |
| *A. desouzai* | 13% | 13% | 12% | 2.2% | – | |
| *A. ambiguus* | 13% | 13% | 12% | 2.6% | 1.2% | – |
| gGAPDH | | | | | | |
| *S. culicis* | – | | | | | |
| *S. oncopelti* | 8.0% | – | | | | |
| *S. galati* | 7.0% | 6.0% | – | | | |
| *A. deanei* | 15% | 12% | 13% | – | | |
| *A. desouzai* | 13% | 11% | 11% | 8.0% | – | |
| *A. ambiguus* | 14% | 12% | 11% | 7.0% | 4.0% | – |
| ITS rDNA | | | | | | |
| *S. culicis* | – | | | | | |
| *S. oncopelti* | 67% | – | | | | |
| *S. galati* | 65% | 45% | – | | | |
| *A. deanei* | 69% | 68% | 57% | – | | |
| *A. desouzai* | 68% | 68% | 60% | 50% | – | |
| *A. ambiguus* | 70% | 73% | 68% | 56% | 35% | – |
| Endosymbiont | | | | | | |
| | *Ca*. K. blastocrithidii | *Ca*. K. oncopeltii | *Ca*. K. galatii | *Ca*. K. crithidii [a] | *Ca*. K. desouzaii | |
| 16S rDNA | | | | | | |
| *Ca*. K. blastocrithidii | – | | | | | |
| *Ca*. K. oncopeltii | 1.4% | – | | | | |
| *Ca*. K. galatii | 1.2% | 1.2% | – | | | |
| *Ca*. K. crithidii [a] | 2.5% | 3.0% | 3.0% | – | | |
| *Ca*. K. desouzaii | 3.0% | 3.0% | 3.0% | 1.4% | – | |
| ITS rDNA | | | | | | |
| *Ca*. K. blastocrithidii | – | | | | | |
| *Ca*. K. oncopeltii | 11% | – | | | | |
| *Ca*. K. galatii | 9.0% | 10% | – | | | |
| *Ca*. K. crithidii[a] | 25% | 26% | 26% | – | | |
| *Ca*. K. desouzaii | 24% | 24% | 24% | 18% | – | |
| *Ca*. K. crithidii[b] | 26% | 26% | 26% | 2.7% | 19% | |

[a] *Ca.* K. crithidii of *A. deanei*
[b] *Ca.* K. crithidii of *A. ambiguus*

The genus *Angomonas* comprises the subgroups deanei and desouzai. The subgroup deanei, in addition to *A. deanei* comb. nov., comprises former *H. roitmani* and four isolates: TCC263E, 1445, 1743 and 1756. The sequences of SSU rRNA from *A. deanei*, *H. roitmani* and all of the isolates of this subgroup are identical. The gGAPDH sequences are very similar among *A. deanei*, *H. roitmani* and TCC263E although slightly divergent from those of the isolates TCC1445 and 1756. The subgroup desouzai comprises *A. desouzai* comb. nov. and the isolates TCC1279 and 1429, which share highly similar sequences, and the new genotype TCC1780, which diverges from these isolates by ~1.2% and ~4.0% for the SSU rRNA and gGAPDH genes. We are designating isolate TCC1780 a new species, *A. ambiguus* sp. nov. (see Taxonomy Section). Sequences of SSU rRNA and gGAPDH genes diverged, respectively ~2.2% and ~8.0% between *A. deanei* and *A. desouzai*, and ~2.6% and ~7.0% between *A. deanei* and *A. ambiguus* (Table 2).

## Polymorphism and Intrageneric Relationships in *Strigomonas* and *Angomonas* by Analysis of ITS rDNA Sequences

Preliminary, analysis of length polymorphisms of the PCR-amplified ITS rDNA (ITS1+5.8S+ITS2) permitted a clear separation between the genera *Angomonas* and *Strigomonas* while revealing species-specific profiles within each genus (data not shown). Thereafter, we sequenced the polymorphic ITS1 rDNA of SHTs to search for genetic polymorphisms that were not detected in the conserved SSU rRNA and gGAPDH genes. Large divergences of ITS1 rDNA sequences (~66%) confirmed the separation of *Strigomonas* from *Angomonas* and revealed high heterogeneity within each genus (Fig. 2 B). Within the genus *Strigomonas,* largest divergences of ~65% and ~67% separated *S. culicis* from *S. galati* and *S. oncopelti* respectively, whereas ~45% of divergence separated the last two species. Relevant divergences also separated the *Angomonas* species: ~50% between *A. deanei* and *A. desouzai*, ~56% between *A. deanei* and *A. ambiguus* and ~35% between *A. desouzai* and *A. ambiguus* (Table 2). In addition, polymorphisms in the ITS rDNA sequences disclosed divergences within the isolates of *A. desouzai* (~10%) and *A. deanei* (~13%).

## Phylogenetic Relationships of Trypanosomatid Proteobacterial Endosymbionts (TPEs)

The presence of symbionts in all of the trypanosomatids nutritionally selected was confirmed by Transmission Electron Microscopy (Figs 6, 7) and PCR amplification of 16S rRNA of the symbionts. Partial 16S rRNA sequences (~800 bp) were determined for the TPEs of all the 13 trypanosomatids studied and aligned with corresponding sequences from closest free living and symbiotic Betaproteobacteria selected by Blast Search from Genbank. Species of Alphaproteobacteria and Gammaproteobacteria were also included in the alignment. Phylogenetic analysis strongly supported the positioning of all TPEs in one monophyletic assemblage within the Betaproteobacteria (Fig. 2A). The phylogenetic analysis accommodate the symbionts in two clearly distinct groups corresponding to the genera *Strigomonas* and *Angomonas*, separated by ~3.3% of sequence divergence. Sequence divergence and positioning in the phylogenetic trees supported five different TPEs (Fig. 2C). Symbionts of isolates of the same SHT species clustered strongly together and exhibited minimal (less than 0.1%) sequence divergences. The symbionts of *A. deanei* and *A. ambiguus*, although belonging to different host species, also presented small divergences (~0.15%) indicating that they belong to the same species of Betaproteobacteria.

Entire 16S rRNA gene sequences (1450 bp) were determined for the five TPE evidenced to be distinct by similarity analysis of partial 16S rRNA sequences. Within the genus *Strigomonas,* the divergences between the entire sequences of 16S rRNA of the symbiont from *S. culicis* and the symbionts of *S. oncopelti* and *S. galati* were 1.4% and 1.2%, respectively. The symbionts of the last two flagellates diverged 1.2% in the whole sequences of 16S rRNA. Within the genus *Angomonas*, the whole 16S rRNA sequences of the symbiont of *A. deanei* diverged 1.4% from sequences of the symbiont of *A. desouzai* (Table 2). Du et al. (1994a,b) had found identical 16S rRNA sequences for the symbionts of *C. oncopelti, C. deanei* and *C. desouzai,* which they assigned to a single Betaproteobacteria species, *Kinetoplastibacterium crithidii*, which does not conform with the present data (see Discussion).

The polymorphism and phylogenetic analyses of both partial and entire 16S rRNA gene sequences permitted the classification of all TPE into the Betaproteobacteria and the separation of the symbionts of *Strigomonas* spp. from those of


**Figure 2.** (**A)** Maximum likelihood (ML) analysis of the 16S rRNA sequences (800 characters, Ln=-3402.734742) of trypanosomatid proteobacterial endosymbionts and symbionts assigned to alpha-, beta- and gamma- Proteobacteria from other organisms retrieved from Genbank. Sequences from free-living species of proteobacteria (▲) were also included in the analysis. Parity analysis of phylogenetic trees based on parsimony (P) analyses of ITS rDNA sequences from SHTs **(B)**, 298 characters, 262 parsimony informative, 433 steps, CI= 0.86, RI= 0.94) and their endosymbionts **(C)**, 586 characters, 170 parsimony informative, 218 steps, CI= 0.93, RI= 0.96). Values indicated at the major nodes correspond to bootstrap support derived from 500 replicates for P and ML, respectively.

*Angomonas* spp. In addition, these analyses distinguished five TPEs despite their very close phylogenetic relationships. To better characterize the TPEs within each genus we performed additional analyses as recommended by Stackebrandt and Ebers (2006). With this purpose, sequences of the more polymorphic ITS rDNA were determined for all the five TPEs already distinguished by 16S rRNA analysis. The sequences were used to make the inferred phylogenetic tree of Figure 2C. Results confirmed the separation of the TPEs into two strongly supported clades separated by an average of 25% of divergences between the symbionts of the genera *Strigomonas* and *Angomonas*. The symbiont of *S. oncopelti* diverged ~11% and ~10% respectively from the symbionts of *S. culicis* and *S. galati,* whereas the symbionts of the last two flagellates diverged ~9.0%. The symbiont of *A. deanei* diverged ~18% and ~2.7% from the symbionts of *A. desouzai* and *A. ambiguus* respectively while the symbionts of the last two hosts diverged ~19%. Divergences of ITS rDNA sequences from the symbionts of the various isolates of *A. deanei* or *A. desouzai* ranged from 0.0% to 3.0%.

The ensemble of sequence analyses and the inferred phylogenetic trees (Fig. 2A, C) permitted the classification of all of the examined TPEs as *"Candidatus"* (as recommended by Murray and Stackebrandt 1995) placing them in the genus Kinetoplastibacterium (Du et al. 1994a). Our results demonstrated that the symbionts of *A. desouzai, A. deanei, S. culicis, S. oncopelti* and *S. galati* are distinct from each other and separated by large genetic distances. Small divergences between the symbionts of *A. ambiguus* and *A. deanei* do not support their separation as distinct TPEs. Taking into consideration the 16S and ITS ribosomal DNA sequence divergences, we are proposing that the TPEs be classified in five distinct species. The symbionts of *A. deanei* and *S. culicis* should retain their original denomination of "*Candidatus* Kinetoplastibacterium crithidii" and *Ca.* K. blastocrithidii whereas the symbionts of *S. oncopelti, S. galati* and *A. desouzai,* should respectively become the new species "*Ca.* K. oncopeltii", "*Ca.* K. galatii" and "*Ca.* K. desouzaii" (see Taxonomy Section).

## Codivergent Host-Symbiont Evolution

The TPEs constitute a well-defined evolutionary lineage within the Betaproteobacteria (Fig. 2A). They are clearly separated from their closest relative free-living Betaproteobacteria *Bordetella avium* by 4.2% - 4.6% divergences in partial 16S rRNA gene sequences. The phylogenetic tree also demonstrated that the clade of TPEs strongly diverged (~7.0 to 15%) from the betaproteobacterial endosymbionts of acanthamoebae (Heinz et al. 2007; Horn et al. 2002), fungi (Bianciotto et al. 2003) and ciliates (Springer et al. 1996). This phylogenetic analyses concurred with the postulated ancient and common origin for all TPEs, thus suggesting that the ability to exploit trypanosomatids as hosts has occurred only once in the evolutionary history within the ancestor of the genera *Angomonas* and *Strigomonas* (Du et al. 1994a).

Congruence analysis between the ITS rDNA-based phylogenetic trees of symbionts and respective trypanosomatid hosts suggest overall host-symbiont co-divergence over evolutionary time. While total congruence was observed at SHT generic level, only partial congruence was found within species of both genera *Angomonas* and *Strigomonas*. The symbiont of *S. oncopelti* and *S. culicis* are the more distant (11% divergence) and their hosts are also the more distant (67%) within the *Strigomonas*. The symbiont of *A. ambiguus* is closet to *A. deanei* (2.7%) than to *A. desouzai* (19%) while the host, *A. ambiguus*, is closer to *A. desouzai* (35%) than to *A. deanei* (56%). Considering the probable common origin of all TPEs, these findings indicate that symbionts and their trypanosomatid hosts are evolving at different evolutionary rates.

## kDNA Minicircle Polymorphisms

Analysis of the classes of kDNA minicircle molecules of SHTs revealed distinct electrophoretic patterns between the genera *Strigomonas* and *Angomonas,* as well as species-specific patterns within each genus (Fig. 3). All of the species of *Strigomonas* displayed complex band patterns. Four and five kDNA bands of different sizes were observed in *S. culicis*, *S. oncopelti* and *S. galati* (Fig. 3). In all species of *Angomonas* two kDNA bands larger than 4.0 kb were found. The largest minicircles belonged to *A. ambiguus* followed by *A. desouzai* (Fig. 3).

The results of this study are consistent with those previously reported for *C. oncopelti* (Pestov et al. 1990), *H. roitmani*, *A. deanei* and *A. desouzai* (Brandão et al. 2000; Hollar et al. 1998; Teixeira et al. 1995), despite the small minicircle size differences due to distinct electrophoretic conditions. However, the findings of these studies together indicate that kDNA minicircles of *Angomonas* comprise two major classes of molecules that are larger than 4.0 kb, whereas those of *Strigomonas* contain

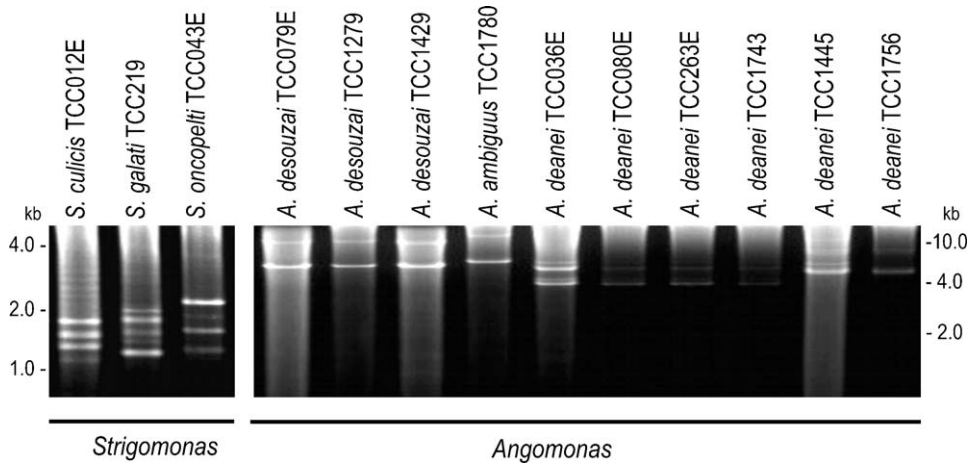


**Figure 3.** Size polymorphism analysis of minicircle molecules of kinetoplast DNA (kDNA) from symbiont-harboring species mechanically decatenated, separated by electrophoresis in agarose gel and stained with bromide ethidium.

at least four classes that are smaller than 3.0 kb (Fig. 3).

## Morphology of SHTs by Optical and Scanning Electron Microscopy

Optical and scanning microphotographs (SEM) of *Strigomonas* and *Angomonas* revealed marked polymorphisms in the culture forms (Figs 4, 5). A morphometric comparison was conducted using optical microscopy of Giemsa-stained logarithmic culture forms of SHT species.

Comparatively, flagellates of *Strigomonas* (Fig. 4) are larger with long flagella whereas flagellates of *Angomonas* (Fig. 5) are smaller and with short flagella. The polymorphism is greater in *Strigomonas* than in *Angomonas,* particularly due to the presence of an undulating membrane in *S. culicis* and campanulate forms in *S. galati*. The proportion of opisthomorphs varies according to the growth phase, they are more abundant in stationary cultures of *Angomonas* (~50 to 70%) than in those of *Strigomonas* (~10% in *S. oncopelti* and ~30% in *S. galati*). They were not seen in cultures of *S. culicis*.

The morphological traits of SH trypanosomatids seem to be species-related. *S. oncopelti* (Fig. 4) displays conical flagellates with pointed posterior ends and truncated anterior extremities. *S. galati* displays a higher level of pleomorphism in shape and size and exhibits the unique morphotype of campanula-like flagellates with a constricted neck at the anterior extremity and a deeply stained ring around the emergence of the flagellum. *S. culicis* displays characteristic undulating membranes, although not as conspicuous as the undulating membranes of trypanosomes. The symbionts of *S. culicis*, called diplosomes in the old literature, can sometimes be seen under light microscopy (Fig. 4). Flagellates of the deanei group are the smallest and most homogeneous SHTs, they have rounded posterior ends and short free flagella. Flagellates of *A. ambiguus* are heterogeneous in shape and size, varying from small and rounded forms with short flagella to large forms with thin posterior ends and long flagella that differ from both the shorter forms observed in the deanei subgroup and the longer forms in the desouzai subgroup (Fig. 5).

We also conducted a comparative SEM analysis between the species of *Strigomonas* and *Angomonas* spp. to illustrate the unexplored morphological diversity of SHT species (Figs 4, 5). The flagellates, especially in the *Strigomonas* spp., displayed twisted bodies with striations on the cell surface that sometimes turned into deep grooves. The flagella in both genera often exhibited lamellae near the emergence from the flagellar pocket. The scanning morphology corroborated the morphological findings revealed by optical microscopy. *Strigomonas* spp. showed a predominance of large forms with thin posterior extremities that were more clearly observed in *S. oncopelti*. The flagellates of *S. galati* displayed forms that had a ring around the emergence of the flagellum. The flagellates of *S. culicis* were the most elongated among all the species of *Strigomonas* and also displayed an undulating membrane. In *A. deanei* small forms predominated with rounded posterior extremities and a short, free flagellum, whereas elongated, twisted-body forms with flagella of variable lengths were observed in *A. desouzai* and *A. ambiguus.*

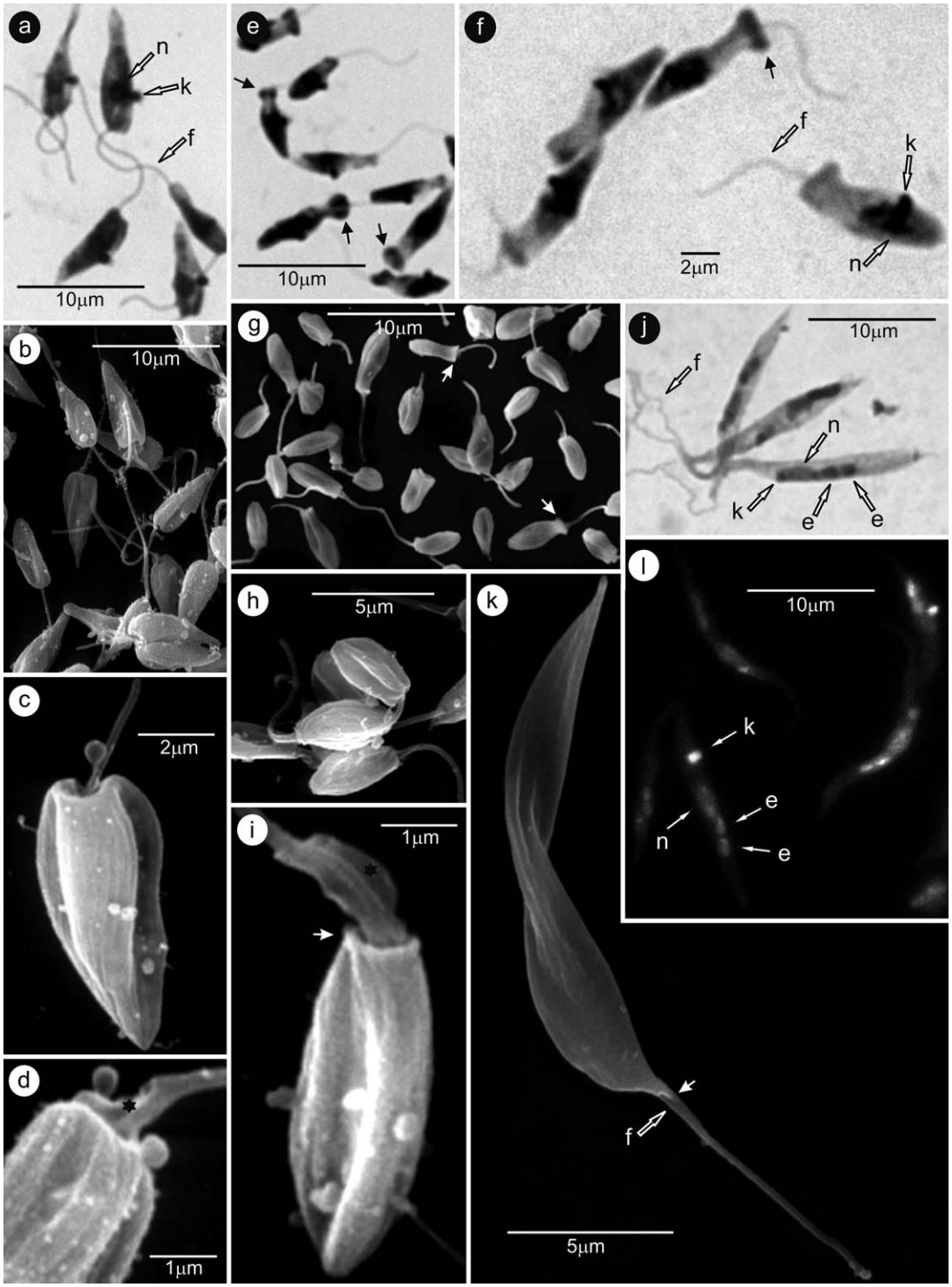


**Figure 4.** Light and scanning microscopy of species of *Strigomonas.* Light microscopy (**a,e,f,j**) and scanning electron microscopy (SEM) (**b,c,d,g,h,i,k,l**) of logarithmic cultures: *S. oncopelti* (a-d), *S. galati* (e-i) and *S. culicis* (j-l). Note the grooves on the cell surface (c,d,h,i), the lamellae in the flagellum (star in d,i) and the typical campanula-like flagellates with a deeply stained ring and constricted neck around the emergence of the flagellum of *S. galati* observed by light microscopy (arrows in e,f) and SEM (arrows in g,i). DAPi staining of *S. culicis* (l). Notice the originally denominated diplosomes (endosymbionts) (j-i) and the poorly developed undulating membrane in *S. culicis* (arrow in k). Nucleus (n); kinetoplast (k); flagellum (f); endosymbiont (e).

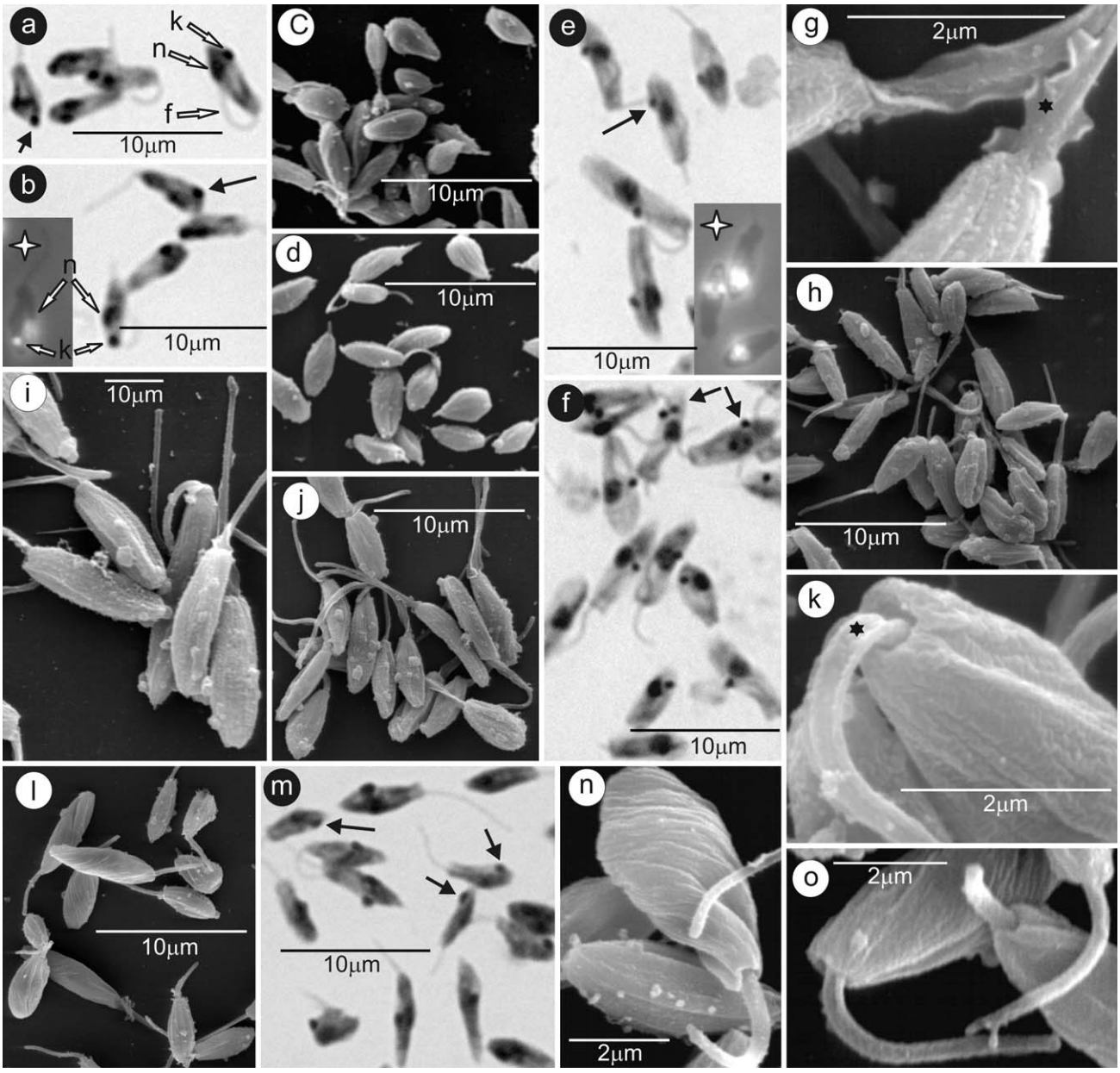


**Figure 5.** Light and scanning microscopy of species of *Angomonas.* Light microscopy (**a,b,e,f,m**) and scanning electron microscopy (**c,d,g-l,n,o**) of log phase cultures of *A deanei* (a,c) and isolates TCC1445 (b) and TCC263E, *A. desouzai* (e,g,h), isolate TCC1279 (f,i,j) and *A. ambiguus* (l,m,n,o). Note the flagellum lamellae signaled by stars (g,k) and the surface grooves (i,n,k). DAPI staining (star) in the inserts (b,e). Nucleus (n), kinetoplast (k), flagellum (f). Black arrows point to opisthomorphs in a,b,e,f,m.

## Morphology of SHTs by Transmission Electron Microscopy

The morphological comparison by transmission electron microscopy (TEM) confirmed the previous observations of SHT ultrastructural features that are common to *A. deanei*, *A. desouzai*, *S. oncopelti* and *S. culicis* (Freymuller and Camargo 1981). In this study, we compared the TEM features of the new species *S. galati* and *A. ambiguus* and the new genotypes of *A. deanei* and *A. desouzai*. The TEM of *S. culicis* has also been fully documented elsewhere (Cavalcanti et al. 2008; De Souza and Motta 1999; Freymuller and Camargo 1981).

Analyses of the ultrastructural cell organization confirmed the presence of peculiarities that are

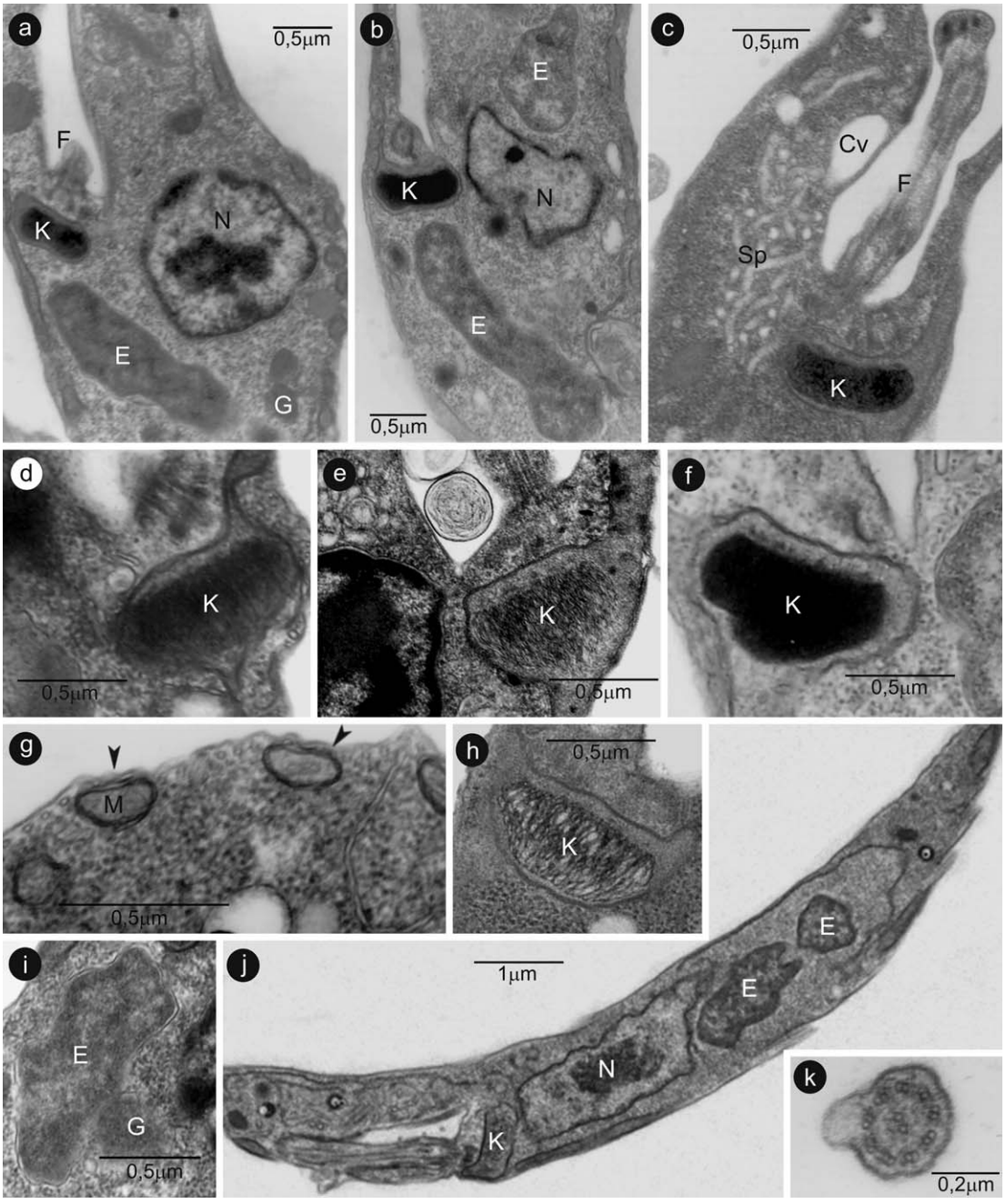


**Figure 6.** Transmission electron microscopy of species of *Strigomonas.* Transmission electron microscopy of *S. oncopelti* (**a,d**), *S. galati* (**b,c,e,f**) and *S. culicis* (**h,j**). Kinetoplasts of *S. oncopelti* (a,d), *S. galati* (b,c,e,f) and *S. culicis* (h) showing a variable compactness and thickness of the kDNA disk. The disks are wider at the center than at the extremities (d,e,f,h). Note the absence of subpellicular microtubules at sites where branches of mitochondrion are appressed to the cell membrane (arrows in g) and the absence of paraflagellar rods (k). Symbionts are enveloped by two membrane units (i), elongated and irregular (a,b,i,j). Nucleus (N), kinetoplast (K), endosymbiont (E), flagellum (F), contractile vacuole (Cv), spongiome (Sp), mitochondrion (M), glycosome (G).

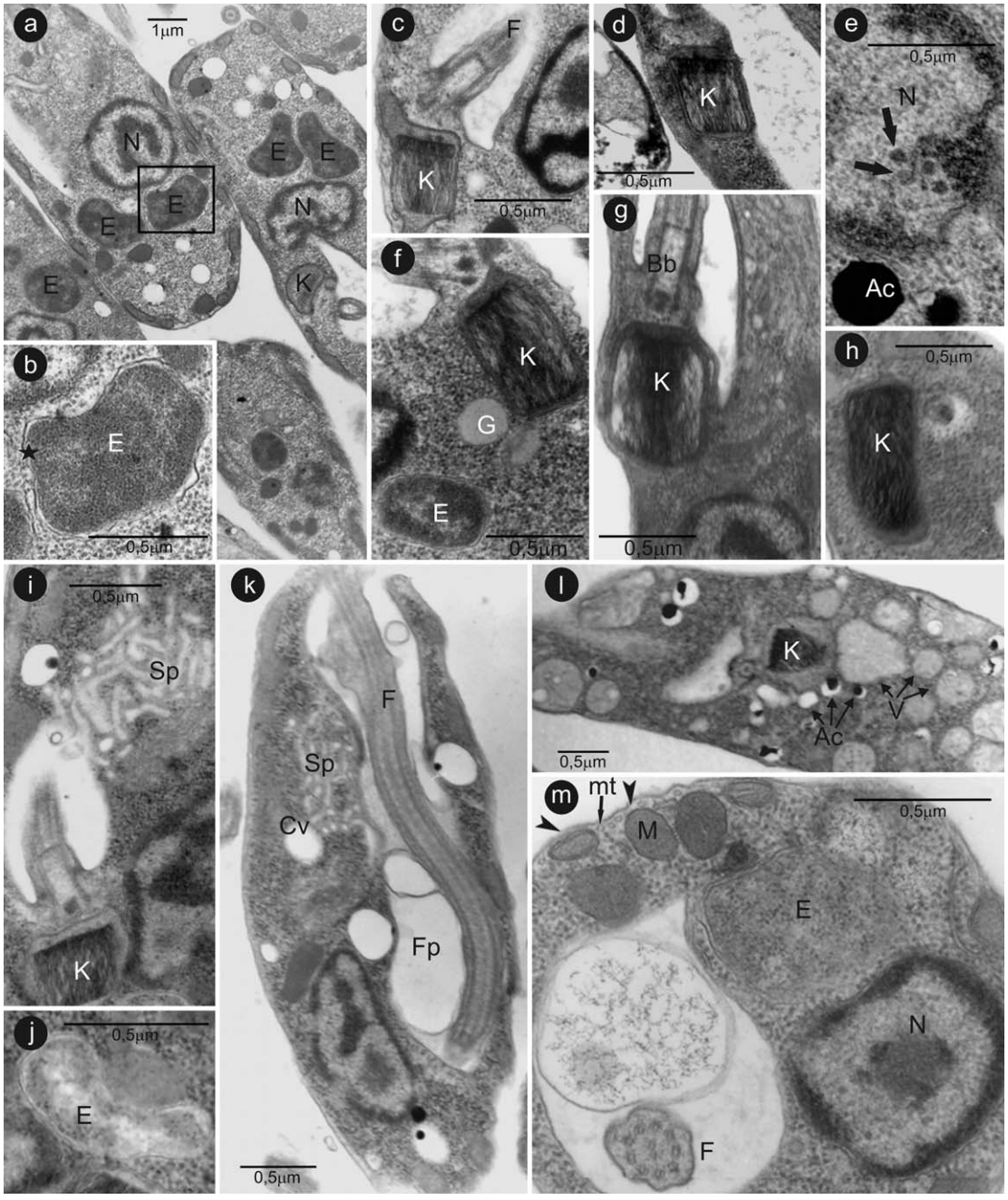


**Figure 7.** Transmission electron microscopy of species of *Angomonas.* Transmission electron microscopy of *A. deanei* (**a,d**), *A. desouzai* (**e,f**), *A. ambiguus* (**b, h-m**), and the new isolates of *A. desouzai* TCC1279 (**g**) and *A. deanei* TCC263E (**c**). Two symbionts in the cytoplasm of *A. deanei* (a) and a dividing symbiont in *A. ambiguus* (j). Endosymbionts are enveloped by two membranes (b,j). Kinetoplasts display a very loose kDNA fibril arrangement in *A. desouzai* (f), isolate TCC1279 (g), *A. ambiguus* (h,i), *A. deanei* (c) and the isolate TCC263 (d) with a characteristic transversal electron-dense band in the last two flagellates. Virus-like particles in *A. desouzai* (arrows in e). Longitudinal section of an opisthomorph of *A. ambiguus* displaying an internal flagellum extending through the length of the cell body (k). Nucleus (N), kinetoplast (K), endosymbiont (E), flagellum (F), glycosome (G), contractile vacuole (Cv), spongiome (Sp), acidocalcisome (Ac), mitochondrion (M), flagellar pocket (Fp), microtubules (mt), basal bodies (Bb).

common to all SHT flagellates (Figs 6, 7): the absence of sub-pellicular microtubules at sites where peripheral branches of the mitochondrion are appressed to the cell membrane, the presence of a bacteria-like cytoplasmic symbiont, one per cell or two in dividing flagellates, the loose arrangement of kinetoplast fibrils and the absence of a typical paraflagellar rod structure. Virus-like particles were only observed in *A. desouzai*, consistent with its original description (Motta et al. 1991). In the opisthomorphs, an internal flagellum extended for the length of the cell body.

SHT flagellates also exhibit features common to all trypanosomatids, such as Golgi complexes, flagella exhibiting the typical array of one central and nine pairs of peripheral microtubule doublets, acidocalcisomes, glycosomes, lipid inclusions, well-developed contractile vacuole/spongiome complex and a single mitochondrion that is located peripherally (Figs 6, 7). However, noticeable differences in the kDNA arrangement result in kinetoplast disk thicknesses of variable sizes and shapes, which are smaller and typically more compact in *Strigomonas* than in *Angomonas.* In *Strigomonas*, the kDNA disk exhibits extremely variable degrees of compactness and thickness and the kinetoplast sometimes appears as bow-shaped (Fig. 6). The average thickness of the kDNA disk is $290 \pm 53$ nm (218-419 nm) in *S. oncopelti,* $270 \pm 83$ nm (148-438 nm) in *S. galati* and $300 \pm 85$ nm (160-480 nm) in *S. culicis*. The kinetoplast disks in *Strigomonas spp.* appear wider at the center than at the extremities (Fig. 6). The kinetoplasts in *Angomonas* appear more or less trapezoid and display very loose kDNA fibril arrangements. In *A. deanei* and the corresponding isolates, the kDNA disk thickness averages $400 \pm 22$ nm (376-419 nm) and exhibits a visible transversal electron dense band (Fig. 7). In *A. desouzai* and the corresponding isolates, the kDNA disk thickness averages $681 \pm 85$ nm (600-809 nm), whereas in *A. ambiguus,* it averages $721 \pm 98$ nm (663-835 nm). In general, the kDNA disk thickness roughly reflects the size of the minicircle classes determined by agarose gel electrophoresis, the larger the minicircle molecules, the thicker and looser are the kDNA arrangements. The symbionts of all *Angomonas* and *Strigomonas* species are surrounded by two membranes separated by an electron-lucent space. Symbionts undergoing division may be observed in logarithmic cultures. Although the symbionts of *Angomonas* and *Strigomonas* exhibit differences in shape, these morphological differences lack diagnostic value due to the marked pleomorphisms apparently related to cell cycle phases.

## Taxonomy Section: Amendments and New SHT Species

**Taxonomic positioning:** Phylum Euglenozoa Cavalier-Smith, 1981, Class Kinetoplastea Konigberg, 1963, Order Trypanosomatida (Kent 1880) Hollande, 1952, Family Trypanosomatidae Doflein, 1951.

**Genus *Strigomonas*** Lwoff and Lwoff 1931.

The type species of the genus *Strigomonas* is *S. oncopelti*. This genus comprises flagellates of variable shapes and lengths in some cases with long flagella accompanied by an undulating membrane. They harbor an irregularly shaped obligate intra-cytoplasmic betaproteobacterial symbiont. The minicircle molecules comprise multiple size classes that are smaller than 3.0 kb. The TEM compactness of the kDNA fibrils is variable and the kinetoplast disks are wider at the center than at the extremities. The genus is phylogenetically defined by constituting a monophyletic cluster within the clade of SHTs according to SSU rRNA, ITS rDNA and gGAPDH genes.

***Strigomonas oncopelti*** Lwoff and Lwoff 1931.

**Synonymy**: *Herpetomonas oncopelti* Noguchi and Tilden 1926, *Leptomonas* (*Strigomonas*) *oncopelti* Lwoff and Lwoff 1931, *Crithidia oncopelti* Gill and Vogel (1963). **Type material**: Culture deposited in ATCC under accession number 12982 for which TCC043E is the duplicate hapantotype deposited in the Trypanosomatid Culture Collection of the University of São Paulo. **Host and locality**: Host unknown, possibly *Oncopeltus* sp., locality, east coast of the USA. **Description:** Culture forms with body lengths from 6.6 to 12 μm (average $8.3 \pm 1.4$ μm), body widths from 2.0 to 3.3 μm ($2.5 \pm 0.3$ μm), and flagellum lengths from 9.0 to 16.0 μm ($12 \pm 2.2$ μm). Stationary cultures display ~10% opisthomorphs. Kinetoplast disk thickness: From 218 to 419 nm ($290 \pm 53$ nm). **Diagnosis:** sequences in GenBank. SSU rRNArRNA: AF 038025 (Hollar and Maslov 1999), gGAPDH: EU079134 (Yurchenko et al. 2009), and ITS rDNA: HM593041/42.

***S. oncopelti* endosymbiont***: "Candidatus* Kinetoplastibacterium oncopeltii" Teixeira and Camargo sp. nov. **Type material**: Obligate symbiotic Betaproteobacteria in the cytoplasm of *S. oncopelti*. **Diagnosis:** Sequences deposited in GenBank. 16S rRNA: HM592997, ITS rDNA: HM593045. **Comments**: This symbiont was originally thought to be identical to the symbiont of *C. deanei* and was mistakenly designated *Kinetoplastibacterium crithidii* by Du et al. (1994b). **Etymology:** "oncopelti" refers to the name of the trypanosomatid host species.

***Strigomonas culicis*** (Wallace and Johnson, 1961) Teixeira and Camargo comb. nov. **Synonymy:** *Herpetomonas culicis* Novy et al. 1907*, Blastocrithidia culicis* Wallace and Johnson 1961**. Type material:** Culture ATCC 30257 of which TCC012E is the duplicate hapantotype deposited in the Trypanosomatid Culture Collection of the University of São Paulo. **Host and locality**: *Aedes vexans,* Diptera Culicidae collected in Mound, Minnesota, USA. (Wallace and Johnson, 1961). **Description:** Polymorphic flagellates with an undulating membrane following the emergence of the flagellum (Fig. 4), body lengths from 15 to 25.70 μm (average $19.30 \pm 2.82$ μm), body widths from 1.2 to 1.9 μm ($1.51 \pm 0.23$ μm), and free flagellum length from 4.2 to 10.5 μm ($7.0 \pm 1.8$ μm). Kinetoplast disk with variable thickness: from 160 to 480 nm ($300 \pm 85$ nm). **Diagnosis:** Sequences deposited in Genbank: SSU rRNA: HM593009, gGAPDH: EU079137, ITS1 rDNA: HQ594516, HQ594517.

***S. culicis endosymbiont***: *"Candidatus* Kinetoplastibacterium blastocrithidii" Du et. al. 1994. **Type material**: Obligate symbiotic Betaproteobacteria in the cytoplasm of *S. culicis*. **Diagnosis:** Sequences in Genbank: 16S rRNA - ITS rDNA: L29265

***Strigomonas galati*** Teixeira and Camargo sp. nov. **Type material:** We are designating the culture TCC219 as the hapantotype, which is deposited in the Trypanosomatid Culture Collection of the University of São Paulo. **Host and locality**: *Lutzomyia almerioi,* Diptera Phlebotomidae collected in Serra da Bodoquena, Mato Grosso do Sul State, Brazil, S20 31 W56 43 (Galati et al. 2006). **Description:** Polymorphic flagellates and peculiar campanulate forms (Fig. 4), body lengths from 3.4 to 10.0 μm (average 6.0 ± 1.2 μm), body widths from 1.3 to 2.8 μm (1.8 ± 0.6 μm), and flagellum lengths from 3.0 to 7.7 μm (5.2 ± 1.4 μm). Kinetoplast thickness: From 148 to 438 nm (270 ± 83 nm). Opisthomorphs: ∼30% in stationary cultures. **Diagnosis:** Sequences deposited in Genbank: SSU rRNA: HM593010, gGAPDH: HM593018, ITS1 rDNA: HM593025/26. **Etymology:** noun in apposition to honor Eunice Galati, a distinguished Brazilian entomologist.

***S. galati endosymbiont***: *"Candidatus* Kinetoplastibacterium galatii" Teixeira and Camargo sp. nov. **Type material**: Obligate symbiotic Betaproteobacteria in the cytoplasm of *S. galati*. **Diagnosis:** Sequences deposited in Genbank: 16S rRNA HM592998, ITS rDNA: HM593046. **Etymology:** "galatii" refers to the name of the trypanosomatid host species.

**Genus *Angomonas*** Souza and Corte-Real, 1991.

The species type of the genus is *A. deanei*, formerly named *Crithidia deanei.* This genus comprises small, rounded flagellates that harbor bacterial endosymbionts and present truncated anterior ends and a large number of opisthomorphs. They harbor an irregularly shaped obligate intra-cytoplasmic betaproteobacterial symbiont. Species within these genera have two classes of minicircle molecules that are larger than 4.0 kb, a minicircle network that displays an extremely loose arrangement and a kDNA disk thickness that is larger than ∼400 nm based on TEM analysis. The genus is phylogenetically defined as a monophyletic cluster within the clade of SHTs according to SSU rRNA, ITS1 rDNA and gGAPDH genes.

***Angomonas deanei*** **(**Carvalho 1973) Teixeira and Camargo comb. nov. **Synonymy:** *Crithidia deanei* Carvalho 1973**. Type material,** the hapantotype is culture TCC036E deposited at the Trypanosomatid Culture Collection of the University of São Paulo. It substitutes for the culture ATCC30255, which is no longer available. **Host and locality**: *Zelus leucogrammus*, Hemiptera Reduvidae, collected in Goiania, Goias State, Brazil. **Paratypes:** Cultures TCC080E, 263E (former 263), 1445, 1743 and 1756, the hosts and geographic origins of which are presented in Table 1. **Description:** Small, rounded choanomastigotes with a short flagellum and exhibiting more than 70% of opisthomorphs in stationary cultures. Body lengths range from 3.6 to 5.5 μm (4.3 ± 0.6 μm), body widths from 1.2 to 2.2 μm (1.5 ± 0.2 μm), and flagellum lengths from 1.0 to 4.0 μm (2.7 ± 1.5 μm). The kinetoplast disk thickness ranges from 376 to 419 nm (400 ± 22 nm). **Diagnosis:** Sequences deposited in Genbank: SSU rRNA: HM593011, gGAPDH: HM593022, ITS1 rDNA: HM593033/34. **Comments:** a) The former *Herpetomonas roitmani* (TCC080E) is identical to *A. deanei* according to all of the molecular markers tested and thus falls in its synonymy, b) If the report of Yurchenko et al. (2009) is confirmed and culture ATCC30255 no longer belongs to *C. deanei*, then culture TCC036E (an old replicate of ATCC30255) should be designated as the type material of *C. deanei***.**

***A. deanei* endosymbiont***: "Candidatus* Kinetoplastibacterium crithidii*"* Du et al. 1994a. **Type material:** Obligate symbiotic Betaproteobacteria present in the cytoplasm of *A. deanei*. **Diagnosis:** Sequences deposited in Genbank: 16S rRNA: HM593003, ITS rDNA: HM593050. **Etymology:** Refers to the name of its trypanosomatid host, which was formerly classified as *Crithidia*.

***Angomonas desouzai*** **(**Fiorini et. al. 1989) Teixeira and Camargo comb. nov**. Synonymy:** *Crithidia desouzai* Fiorini et al. 1989**. Type material:** Culture ATCC50305, for which culture TCC079E is the duplicate hapantotype deposited in the Trypanosomatid Culture Collection of the University of São Paulo. **Paratypes:** Cultures TCC1279 and TCC1429. **Host and locality:** *Ornidia obesa*, Diptera Syrphidae, collected in Alfenas, Minas Gerais State in Brazil. **Description:** Culture forms are choanomastigotes and opisthomorphs (∼40% in stationary cultures). Body lengths from 5.0 to 9.0 μm (average 6.0 ± 1.1 μm), body widths from 1.1 to 2.0 μm (1.6 ± 0.2 μm) and flagellum lengths from 1.7 to 6.0 μm (3.8 ± 2.1 μm). The kinetoplast disk thickness ranges from 600 to 809 nm (681 ± 85 nm). **Diagnosis:** Sequences deposited in Genbank: SSU rRNA**:** HM593016, gGAPDH: HM593020, ITS1 rDNA: HM593027/28. A peculiarity is the presence of symbiotic viral particles in the cytoplasm and nucleus (Motta et al. 1991).

***A. desouzai*** **endosymbiont***: "Candidatus* Kinetoplastibacterium desouzaii*"* Teixeira and Camargo sp. nov. **Type material:** Obligate symbiotic Betaproteobacteria present in the cytoplasm of *A. desouzai*. **Diagnosis:** Sequences deposited in Genbank: 16S rRNA: HM592999, ITS rDNA: HM593047. **Etymology:** Refers to the name of the trypanosomatid host species.

***Angomonas ambiguus*** Teixeira and Camargo sp. nov. **Type material:** We are designating as the hapantotype, the culture TCC1780, which is deposited in the Trypanosomatid Culture Collection of the University of São Paulo. **Host and locality:** *Chrysomya albiceps,* Diptera Calliphoridae, collected in Campo Grande, Mato Grosso do Sul State, Brazil, S19 54 W55 16. **Description:** Choanomastigotes and opisthomorphs about 50% in stationary cultures. Body lengths from 3.0 to 6.6 μm (average 4.9 ± 1.0 μm), body widths from 1.1 to 2.1 μm (1.6 ± 0.26 μm), and flagellum lengths from 1.0 to 8.6 μm (3.7 ± 2.1 μm). The kinetoplast disk thickness ranges from 663 to 835 nm (721 ± 98 nm). **Diagnosis:** Sequences deposited in Genbank: SSU rRNA: HM593015, gGAPDH: HM593019, ITS1 rDNA: HM593031/32. **Etymology:** "ambiguus" meaning ambiguous because of the non-congruent phylogenies between the flagellate host and its symbiont.

***A. ambiguus*** **endosymbiont***: "Candidatus* Kinetoplastibacterium crithidii*",* the same Betaproteobacterial symbiont of *A. deanei.* **Diagnosis:** Sequences deposited in Genbank: 16S rRNA: HM593002, ITS rDNA: HM593049.

## Discussion

The taxonomy of the Trypanosomatidae acquired its current form after Hoare and Wallace (1966) proposed their genera-defining criteria based on

phenotypes and life cycles. These criteria straightened out the prevailing disarray in the taxonomy of trypanosomatids but were not exempt from conceptual and operational problems. One recurring problem resulted from difficulties in the recognition of the morphotypes used to define each genus, which required training and expertise. The problem was complicated because not all of the newly described trypanosomatids fit neatly into the proposed genera (Merzlyak et. al. 2001; Svobodova et. al. 2007). Several mistakes in the taxonomy of SHTs exemplify these problems. Most SHTs have been placed in the genus *Crithidia* because of their choanomastigote-like shape. However, one SHT species was moved from *Crithidia* to *Herpetomonas* because it displayed choanomastigotes with kinetoplasts located in the posterior end that resembled opisthomastigotes (Faria-e-Silva et al. 1991, 1996; Fiorini et al. 1989). These unusual choanomastigotes, present in cultures of *Angomonas* and *Strigomonas,* corresponded to a new morphotype that was later designated opisthomorph (Teixeira et al. 1997).

Other complications resulted from the mixing of cultures and the utilization of wrongly identified samples. For instance, the data presented herein demonstrate that the symbiont "*Ca.* K. crithidii" is exclusive to *A. deanei* and *A. ambiguus*. However, this symbiont has been considered to be common to *S. oncopelti*, *A. desouzai* and *A. deanei,* because the cultures examined by Du et al. (1994a, b) actually were cultures of *A. deanei* (=*C. deanei*) as evidenced by the nearly identical SSU rRNA sequences of these three species of *Crithidia* (Du and Chang 1994a). In the present study, we confirmed that the SSU rRNA gene sequences reported by Du et al. (1994a) were in fact identical to the sequences of *A. deanei,* but diverge from the sequences of *A. desouzai* and *S. oncopelti*.

Inconsistencies in the identification of SHT cultures are not novel and have been reported in previous studies (Hollar et al. 1998; Teixeira et al. 1995, 1997; Yurchenko et al. 2009). Such was the case for aposymbiotic strains of *C. oncopelti* and *C. deanei* from the ATCC, which presented identical SSU rRNA and gGAPDH sequences, and the presumed ATCC culture of *C. deanei*, which lacked an endosymbiont and was more likely a culture of *C. luciliae thermophila* (Yurchenko et al. 2009). Fortunately, we keep replicates of the ATCC cultures from *C. deanei* and *C. oncopelti*, which were deposited in the Trypanosomatid Culture Collection USP in 1981. The present results obtained for these cultures are in accordance with previous data for the original ATCC samples with respect to morphology (Freymuller and Camargo 1981), kDNA minicircles (Brandão et al. 2000; Camargo et al. 1982; Hollar et al. 1998; Pestov et al. 1990), spliced leader (Fernandes et al. 1997), SSU rRNA (Hollar et al. 1998) and gGAPDH (Hamilton et al. 2004) genes.

Our comparative molecular study harmonizes genetic diversity and phylogenies with the phenotypic singularities of the species nested in the SHT clade, which in addition to a bacterial symbiont displays unique metabolic capabilities and morphological peculiarities. However, metabolism and morphology can vary according to the culture conditions and the growth phase, which limits the value of these parameters for SHT taxonomy. In the present paper, we integrated nutritional, morphological and phylogenetic data to characterize the SHTs.

Barcoding of the variable V7V8 region of the SSU rRNA gene has been confirmed as a powerful tool for the assessment of the genetic diversity of trypanosomatids in general (Ferreira et. al. 2007, 2008; Maia da Silva et al. 2004; Viola et. al. 2008, 2009). In the present study, barcoding was employed to select isolates for inclusion in phylogenies based on independent or combined SSU rRNA and gGAPDH genes, which provided strong evidence of the monophyly of the SHT and its partition in the two subclades corresponding to the genera *Strigomonas* and *Angomonas*. The ensemble of phylogenetic inferences clearly separated *Angomonas* and *Strigomonas* from the genera *Blastocrithidia*, *Crithidia* and *Herpetomonas*, in which they have been traditionally placed. The proposed designation of the new SHT genera was facilitated by their clearly distinctive genotypic characteristics. The genera *Angomonas* and *Strigomonas* could be distinguished from the remaining trypanosomatid genera based on the presence cytoplasmic bacterial symbionts and the ability to grow in hemin-free media. The genera *Strigomonas* and *Angomonas* could be distinguished from each other by kinetoplast morphology, molecular kDNA features and phylogenetic analyses involving the SSU rRNA, ITS1 rDNA and gGAPDH genes. In morphological terms, the genus *Angomonas* is more homogeneous than the genus *Strigomonas.* In molecular terms both genera are clearly monophyletic. The morphological differences between the species of *Strigomonas* reflect the conflict between morphology and phylogeny, which is not new in the taxonomy of the Trypanosomatidae (Hollar et al. 1998, Yurchenko et al. 2008). Phylogeny is proving to be more consistent than morphology for taxa discrimination in trypanosomatids. Accordingly, the unquestionable

monophyly of the group constituted by the former *C. oncopelti*, *B. culicis* and the new species *S. galati* has led us to place them together in the genus *Strigomonas*.

An explanation is necessary about the proposed placement of *B. culicis* in the genus *Strigomonas*. The genus *Blastocrithidia* (Laird 1959) was created over a polyphyletic character, the undulating membrane, which occurs in more than one trypanosomatid taxon. The type species of the genus *Blastocrithidia* is *B. gerridis* (Laird 1959; Patton 1908), which has never been cultured and is not available for comparison. In his revision of the insect trypanosomatid genera, Wallace (1966) put together in the genus *Blastocrithidia* all trypanosomatid species displaying an undulating membrane. However, flagellates classified as *Blastocrithidia* according to the definition of Wallace do not constitute a monophyletic assemblage in molecular phylogenetic analyses in previous works (Hollar et al.1998; Merzlyak et al. 2001; Yurchenko et al. 2008) and in the present paper. Eventually, the genus *Blastocrithidia* will have to be revised. In the meantime, it would be a poor taxonomic practice to place a known polyphyletic taxon into a clearly monophyletic one, the SHT taxon. For this reason we are moving *B. culicis* to the genus *Strigomonas*. The move is fully justified in molecular terms. The SSU rRNA divergences separating the three *Strigomonas* species (~7 - 8%) are much smaller than the divergences separating these species from representatives of the remaining genera of insect trypanosomatids. The SSU rRNA divergences with *Strigomonas* spp. are approximately: *Phytomonas serpens* (19.5%), *Herpetomonas muscarum* (18.5%), *Leptomonas seymouri* (18%), *Crithidia fasciculata* (17.5%), *Wallaceina sp.* (17.5%), *Sergeia podilipaevi* (19%), *Angomonas deanei* (13%) and *Blastocrithidia triatomae* (21%), Actually, *S. culicis* is more distant from *B. triatomae* than to any other of the above listed species, emphasizing the polyphyly of the genus. However, a cautionary word is necessary. The sample examined of *Strigomonas* is too small to permit definitive and absolute taxonomic conclusions. It is conceivable that the examination of a larger number of new isolates may require a revision of the SHT taxon and the spliting of them into new taxa. This may apply mainly to the genus *Strigomonas*, which exhibits considerable morphological and molecular heterogeneity as compared to the genus *Angomonas*.

Concerning species, the criteria adopted for the definition and identification of species of Trypanosomatidae still constitute an unsettled matter. The combined use of morphological and molecular parameters is the best that can be done for species identification at the moment (Maslov et. al. 2010; Svobodova et. al. 2007; Viola et. al. 2009; Yurchenko et. al. 2008, 2009). For the distinction between the three species of *Strigomonas,* morphology provided more consistent information than for the distinction among the species of *Angomonas*. Although morphology did not provide a decisive diagnostic criterion, molecular analyses clearly distinguished three species within *Angomonas: A. deanei*, *A. desouzai* and *A. ambiguus.* In addition, polymorphisms in the kDNA minicircles and ITS rDNA evidenced distinct genotypes in *A. deanei,* two isolates being identical to and two slightly different of the heading species.

Phylogenetic inferences based on 16S rDNA and in the ITS rDNA sequences of the symbionts of *Strigomonas* and *Angomonas* disclosed five species and strongly supported their classification in the monophyletic clade of the Betaproteobacteria, as previously demonstrated for the symbionts of *A. deanei* and *S. culicis* (Du et al. 1994a).

The genomes of obligate endosymbionts markedly differ from those of free-living organisms exhibiting a reduced size, a biased AT content and faster evolutionary rates. Endosymbionts and hosts are mutually dependent. This inter-dependency results in the inability of the symbiont to grow outside of the host cell and a dependence of the host on the symbiont for many functions (Moran et al. 2008; Moran and Plague 2004; McCutcheon and Moran 2010). A long co-evolutionary history between trypanosomatid hosts and symbionts is supported by the finding that aposymbiotic SHTs are difficult to culture and that TPEs are obligate symbionts that do not survive outside their hosts (Roitman and Camargo 1985). Regardless of the unknown mechanisms and timing and of the symbiotic association, hosts and symbionts share a common and ancient evolutionary history, which was corroborated in the present study by a comparison of the phylogenies of SHTs and their symbiotic bacteria. The results revealed an overall congruence between host and symbiont phylogenies, with complete parity at genus level. One exception at species level involved *A. ambiguus*, which is closely related to *A. desouzai*, but whose symbiont is phylogenetically closer to *A. deanei*.

Forthcoming knowledge concerning the interaction between SHTs and their symbionts may lead to a better understanding of their co-divergent history and the conversion of free-living Proteobacteria into obligate symbionts during the origin of eukaryotic cell organelles. Unfortunately, the avail-

able data on SHT species are still too restricted to clarify many aspects of their evolutionary history including host-species association and ecological and phylogeographical patterns. Most of the SHTs characterized in the present study, which comprises all new and previously reported species, belong to *Angomonas,* and all of them originated from the digestive tract of Diptera from Brazil, with the exception of *A. deanei,* which was obtained from a predator hemipteran. However, flies and not hemipterans may be the habitual hosts of *Angomonas* as suggested here by the fact that all new isolates came from flies. An analysis of a larger sample of SHTs, including representatives of distinct insect hosts from different continents, remains necessary to substantiate our knowledge concerning the taxonomy and the evolution of SHT flagellates and the symbiotic partnerships between Proteobacteria and trypanosomatids.

## Methods

**Isolation and culture of new flagellates:** The isolate of *C. oncopelti* used in this paper (TCC043E) was obtained from the ATCC (12982) and from F. G. Wallace in 1981. *C. deanei* (TCC036E) was also from the ATCC (30969) and was received from I. Roitman in 1982. *C. desouzai* and *H. roitmani* were received from J. E. Fiorini in 1989. In this paper, *C. oncopelti* was reclassified as *S. oncopelti* whereas *C. deanei* and *C. desouzai* were reclassified in the genus *Angomonas* (see Taxonomy Section). New isolates were obtained from the digestive tracts of Diptera, Calliphoridae, collected in southeastern, central and southern Brazil. The isolate TCC219 came from Psychodidae fly collected in Central Brazil during a *Leishmania* spp. survey (Galati et al. 2006). After the removal of contaminating fungi and bacteria, the trypanosomatid cultures were cloned as described previously (Sbravate et al. 1989). All of the reference species and new flagellates are listed in Table 1, and they are all cryopreserved in the Trypanosomatid Culture Collection (TCC) of the University of São Paulo, São Paulo, Brazil. Preliminarily, we examined the nutritional requirement for heme of ∼500 cultures of trypanosomatids from the TCC-USP collection. Species and isolates of SHTs were cultivated in FYTS (Mundim et al. 1974), LIT (Camargo 1964) or Grace medium supplemented with 2-5% SFB at 25-28 °C. For the nutritional selection of SHTs, flagellates were cultivated in hemin-free LIT medium for at least 10 successive passages. The generation times were scored for representative SHTs.

Aiming at the axenic cultivation of the symbionts, the flagellates were disrupted by mechanical means, and the homogenates were aseptically processed to obtain a fraction that was enriched for symbionts (Alfieri and Camargo 1982). The inoculation of crude homogenates as well of symbiont-enriched fractions in standard microbiological media for Gram-negative and Gram-positive bacteria did not result in positive cultures during the six weeks of observation.

**Morphological analysis by light and electron microscopy:** For light microscopy, smears on glass slides of flagellates cultivated in Grace medium were fixed with methanol and stained with Giemsa and additionally by the DAPI-staining (Motta et al. 2010). Measurements were conducted for 30-50 flagellates in logarithmic-phase cultures from each species. For transmission electron microscopy (TEM), the culture flagellates were fixed in glutaraldehyde, post-fixed with osmium tetroxide, dehydrated in ethanol and embedded in Spurr̈s resin (Viola et al. 2009). Ultrathin sections were stained with uranyl acetate and lead citrate as described previously (Ventura et al. 2000, Viola et. al. 2009) and analyzed using a JEOL 100CX electron microscope. For scanning electron microscopy (SEM), trypanosomatids from the cultures were fixed in 2.5% (v/v) glutaraldehyde containing 1% (w/v) paraformaldehyde in 0.1 M cacodylate buffer (pH 7.2), adhered to 0.1% (v/v) poly-L-lysine-coated coverslips, post-fixed in 1% (w/v) OsO4 in 0.1 M cacodylate buffer for 30 min in the dark, dehydrated in an ascending ethanol series, critical point-dried with $CO_2$ in a Balzers CPD 030 critical point dryer, sputtered with gold in a SCD 050 Balzers sputter coater and observed using a ZEISS DSM 940 digital scanning microscope.

**Phylogenetic inferences based on SSU rRNA and gGAPDH gene sequences:** Total DNA was extracted from cultured trypanosomes using the classical phenol-chloroform method, and the V7V8 region of SSU rRNA, whole SSU rRNA and ITS1 rRNA genes were PCR-amplified using previously described primers and PCR conditions (Ferreira et al. 2007, 2008; Maia da Silva et al. 2004; Viola et al. 2008). The gGAPDH sequences were amplified as previously described (Hamilton et al. 2004; Viola et al. 2009). The sequences determined in this study were deposited in GenBank under the accession numbers listed in the Taxonomic Section. These sequences were aligned with corresponding sequences from other trypanosomatids available in GenBank using ClustalX (Thompson et al. 1997) and manually refined. Four alignments were generated for analysis using parsimony (P), maximum likelihood (ML) and Bayesian inference (BI) methods: A1, consisting of the variable V7V8 SSU rRNA gene region (1018 bp) from all SHTs and other insect flagellates, A2, 1918 bp of the SSU rRNA sequences from all of the different SHTs and from the species of several genera representative of the main clades in the trypanosomatid phylogenetic tree, without regions of ambiguous alignment as described previously (Hamilton et al. 2004; Viola et al. 2009), A3, gGAPDH sequences (864 bp) from the same taxa selected for alignment A2, A4, concatenated SSU rRNA and gGAPDH gene sequences of the same taxa included in alignments A2 and A3 (2782 bp). The alignments used in this study are available from the authors upon request.

The parsimony and bootstrap analyses were performed using PAUP* version 4.0b10 software (Swofford 2002), with 500 random sequence addition replicates followed by branch swapping (RAS-TBR). Bayesian analysis was performed using MrBayes v3.1.2 (Huelsenbeck et al. 2001). The tree searches employed GTR plus gamma and the proportion of invariable sites. The first 25% of the trees from 1,000,000 generations were discarded as burn-in to allow the log-likelihood scores to reach stability. Default values were used for the remaining parameters. ML analyses were performed using the GTR+Γ+I model in RAxML v.7.0.4 (Stamatakis 2006) with 1,000 maximum parsimony starting trees. Model parameters were estimated in RAxML over the duration of the tree search. Bootstrap support was estimated with 1000 replicates in RAxML using the rapid bootstrapping algorithm (Stamatakis et al. 2008) and maximum parsimony as starting trees.

**Polymorphism analysis of ITS1 rDNA from trypanosomatids:** The primers and conditions employed for PCR amplification of whole ITS rDNA (ITS1+5.8S+ITS2) or the segment corresponding to the ITS1 rDNA have been previously described (Ferreira et al. 2007; Maia da Silva et al. 2004). The PCR-amplified products were cloned using the TA Cloning Kit

(Invitrogen), and at least three clones from each flagellate were sequenced.. Sequences were aligned using ClustalX and manually refined. ITS rDNA sequences of other trypanosomatids species were not included in the analysis due to unreliable alignments. Phylogenetic inferences were assessed using parsimony (P) and Bayesian (BI) as described above.

**Determination of the classes of kDNA minicircle molecules:** The length polymorphism of the classes of SHT kDNA minicircle molecules were assessed by 2.5% agarose gel electrophoresis of mechanically decatenated kDNA obtained from the total DNA of flagellates, followed by ethidium bromide staining as described previously (Ventura et al. 2000). The kDNA origin of the visualized bands was confirmed by Southern blot hybridization using a probe complementary to the conserved replication origin of the kDNA minicircles, according to protocol described by Ventura et al. (2000).

**Amplification and data analysis of 16S rRNA and ITS1 rDNA from trypanosomatid symbionts:** PCR amplification of the 16S rRNA gene was performed to confirm the presence of bacteria-like symbionts in all the new and reference isolates compared in this study. The amplification of the 800 bp 16S rDNA fragment was conducted using primers 680F (5'GTAGCAGTGAAATGCGTA3') and 1486R (5'GATACGGCTACCTCCTTACG 3'). The amplification of the entire 16S rDNA sequence was done using primers: p1seq (5'AGAGTTTGATCCTGGCTCAG3') and 1486R (5' GATACGGCTACCTCCTTACG 3'). The PCR reactions contained 100 ng of DNA, 2.5 U of Taq DNA polymerase, 0.2 mM each dNTP and 200 pM of each primer. Thirty cycles were performed as follows: 1 min at 94 °C, 2 min at 60 °C and 2 min at 72 °C. The amplified 16S rRNA fragments (~800 bp) were sequenced and aligned with the corresponding sequences from GenBank of symbiotic $\alpha$, $\gamma$ and Betaproteobacteria and free-living Betaproteobacterial species (Fig. 2A). The ITS rDNA sequences of the symbionts (~500 bp) were also determined using primers p1398F (5'ACACCATGGGAGTGGGTT3') and p23sRev (5'TCCAAGGCATCCACCGTA3') (Du et al. 1994a). The amplifications were performed as described for the 16S rRNA gene but with a 51 °C of annealing temperature. Based on an analysis of the secondary structure, this region likely contains two tRNA genes. Phylogenetic inferences using 16S rRNA and ITS1 rDNA sequences were performed using P and ML as described above for the phylogenetic analyses of trypanosomatids.

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# Insect parasites of the genus *Angomonas*: Phylogeographical evidence of cryptic diversity, parasite-symbiont co-divergence and dispersion by blowflies throughout the Neotropics and Afrotropics

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**Abstract**

We assessed the biogeography and the genetic diversity of symbiont-harboring trypanosomatids (SHTs) of the genus *Angomonas* parasitizing flies and compared the species composition in the Neotropics and Afrotropics. Barcoding using the variable region of V7V8 SSU rRNA (ribosomal small subunit of rRNA gene) of 364 trypanosomatid cultures from flies (~3700 flies examined) collected in South America and Africa identified 70 cultures (~19%) as *Angomonas,* 52 (74%) of them from blowflies. All cultures of *Angomonas* were from flies excepting the reference *A. deanei* that is from a predator hemipteran. Surveys of *Angomonas* directly on blowfly guts by PCR targeting the GAPDH (Glyceroaldehyde Phosphate Dehydrogenase) gene from the trypanosomatid proteobacterial endosymbionts (TPEs) revealed that ~51% of blowflies were infected by *Angomonas* in South America and Africa, and allowed for the characterization of 13 uncultured isolates. From the 83 *Angomonas* isolates characterized, 61 were *A. deanei,* 16 *A. desouzai* and 6 *A. ambiguus.* Phylogenetic analysis using gGAPDH sequences of selected SHT isolates, either from cultures or directly from fly guts, representative of the ample diversity of host and geographical range, corroborated the main clades of the genus *Angomonas,* represented by *A. deanei, A. desouzai* and *A. ambiguus,* and found in South America and Africa. This analysis also disclosed cryptic intra-specific diversity: four genotypes (Ade1-Ade4) within *A. deanei* and two (Ads1-Ads2) in *A. desouzai* while *A. ambiguus* was more homogenous. The phylogenies of the TPEs based on sequences from GAPDH, Rpi (ribose 5-phosphate isomerase A) and ITS rDNA also disclosed four TPE genotypes (SAde1-SAde4) of *A. deanei* symbiont, and two genotypes of *A. desouzai* symbiont. These findings corroborated the SHT-TPE co-divergence inferred from previous studies as well as the sharing of only one TPE by *A. deanei* and *A. ambiguus.* Flies of distinct families harbored the three species of *Angomonas.* Despite the strong association of *Angomonas* with blowflies, these flies also harbour many trypanosomatids of other genera. Calliphorids of the genera *Chrysomya* and *Lucilia* accounted for the majority of isolates (74%) followed by flies of Muscidae (17%). The main hosts of *Angomonas* are *Chrysomya,* regardless from Africa (Guinea Bissau, Mozambique and Madagascar) or South America (Brazil, Venezuela and Colombia) where *Chrysomya* was recently introduced from Africa. Blowflies of the genus *Lucilia* are important hosts of *Angomonas* in Brazil while *Cochlyomyia,* the native blowflies of the Neotropics, show low infection rates. The *Angomonas* species are all cosmopolitan, lacking spatial structure at species and genotype levels, and also lack host-species association. The comprehensive analysis of the tripartite *Angomonas*-symbionts-calliphorids allowed to hypothesize that the recent dispersion of blowflies play an important role in the introduction into the Neotropics and in the worldwide distribution of the species and genotypes of *Angomonas.*

**Keywords**: trypanosomatid, blowfly, insect parasite, codivergence, species diversity, cosmopolitan species, beta-proteobacterian symbionts

## Introduction

The family Trypanosomatidae congregates flagellates of the class Kinetoplastea, phylum Euglenozoa, which parasitize all classes of vertebrates (Hoare, 1972), some plant families (Camargo, 1998) and insects of several orders, mainly Diptera and Hemiptera. Insects are the main hosts of trypanosomatids and also are the main vectors of vertebrate trypanosomes (Hoare & Wallace, 1966; Wallace, 1966).

Species of the insect trypanosomatid of the genera *Strigomonas* and *Angomonas* carry endosymbionts in their cytoplasm and are called SHTs (Symbiont Harboring Trypanosomatids). In phylogenetic trees, SHT species cluster together in a monophyletic assemblage formed by two subclades corresponding to the genera *Angomonas* and *Strigomonas.* The genus *Strigomonas* is represented by three highly divergent species: *S. culicis*, *S. oncopelti* and *S. galati*. The genus *Angomonas* comprises the species *A. deanei, A. desouzai* and *A. ambiguus* (Teixeira et al., 2011).

The obligate symbionts (Trypanosomatid Proteobacterial Endosymbionts, TPEs) of the SHTs belong to the beta division of the Proteobacteria and are classified into five *Candidatus* Kinetoplastibacterium species (Teixeira et al., 2011; Alves et al., 2011; 2013a; Motta et al., 2013). *A. deanei* and *A. ambiguus* carry the same TPE species whereas the TPE of *A. desouzai* is unique of this species. Endosymbionts and hosts are mutually dependent. The symbionts participation in the metabolism of their hosts (Roitman & Camargo, 1985; Motta, 2010), renders the flagellates autotrophic for hemin (Chang et al., 1975; Kořený et al., 2010; Alves et al., 2011), vitamins (Klein et al., *personal communication*) and some amino-acids (Galinari & Camargo 1978, 1979; Alfieri & Camargo, 1982; Motta et al., 2013; Alves et al., *in press*), which are obligatory nutritional requirements for the trypanosomatids. The symbiotic association between hosts and symbionts suggest an ancient evolutionary association corroborated by the congruent SHTs and TPE phylogenies. The exception is *A. ambiguus*, which is closely related to *A. desouzai* but shares the symbiont with *A. deanei* (Teixeira *et* al., 2011; Alves et al., 2013a).

*Strigomonas* and *Angomonas* occur in Diptera and Hemiptera, but dipterans appear to be their commonest hosts of *Angomonas* and the genus *Herpetomonas* (Teixeira et al., 1997, 2011; Borghesan et al., 2013; Týč et al., 2013). We previously characterized 10 isolates of *A. deanei, A. desouzai* and *A. ambiguus*: 7 from Calliphoridae, two from Syrphidae and one from a predator hemipteran of Reduviidae (Teixeira et al., 2011). A recent study on trypanosomatids of flies (Týč et al., 2013) reported the occurrence of these three species of *Angomonas* in 9 flies, three *A. deanei* in Calliphoridae, three in Sarcophagidae and one in Muscidae; one culture of *A. ambiguus* from calliphorid of Papua New Guinea and one of *A. desouzai* from sarcophagid of Ecuador were obtained. The studies on fly trypanosomatid showed a worldwide distribution of *Angomonas* spp. sharing hosts of distinct dipteran families with trypanosomatids of the genus *Herpetomonas* (Teixeira et al., 1997; Borghesan et al., 2013; Týč et al., 2013).

In contrast with the data on the *Herpetomonas*-fly association (Borghesan et al., 2013; Týč et al., 2013), available data on *Angomonas* were too limited for reliable biogeographical analyses. The first species described of *Angomonas* was *A. deanei* in Central Brazil in 1975, before the introduction of *Chrysomya* in Brazil in 1976 (Guimarães et al., 1978, 1979). The host of *A. deanei* was of Reduviidae that, most likely, got infected by feeding on an infected fly since hemipterans are not usual hosts of SHTs (Sbravate et al., 1989; Camargo et al., 1992; Godoi et al., 2002; Yurchenko et al., 2009; Votýpka et al., 2010; 2012; Jirku et al., 2012; Týč et al., 2013).

Calliphoridae (Diptera, Brachycera, Calyptratae) are a cosmopolitan group of flies, which includes species of medical, veterinary, and forensic importance. The origin and timing of the divergence among the extant calliphorids are not settled matters with estimates varying from the breakup of the Gondwana to ~250 mya (Wiegmann et al., 2003; Wallman et al*.,* 2005; Yeates & Wiegmann, 2005; Stevens & Wallman, 2006; Stevens et al., 2006; Bertone et al*.,* 2008; Harvey et al*.,* 2008; Bertone & Wiegmann, 2009; McDonagh & Stevens, 2011; Nelson et al., 2012; Singh & Wells, 2013). Calliphorids have worldwide occurrence, but the calliphorid species present distinct geographic origin and current distribution. In the New World, the main constituents of the Calliphoridae are species of the genera *Cochliomyia* and *Lucilia* and the cosmopolitan species of the genus *Chrysomya,* recently introduced into the Neotropics. The neotropical *Lucilia eximia* had been recorded as the predominant species in Brazil since the years 1930 while the cosmopolitan *Lucilia sericata* was introduced in Brazil shortly before the years 1960 (Mello, 1961). In 1976, the Old Word calliphorids *Chrysomya albiceps*, *Chrysomya megacephala* and *Chrysomya putoria* invaded Brazilian Southern seaports brought by African (Angolan) refugees and their domestic animals, and rapidly spread all over Brazil, and South, Central and North America (Guimarães et al*.,* 1978, 1979; Kurahashi, 1980; Richard & Gerrish, 1983; Wells, 1991; Guimarães & Papavero, 1999; Baumgartner & Greenberg, 1984; Laurence, 1981; Mariluis, 1980; Prado & Guimarães, 1982). The invading *Chrysomya* apparently caused the displacement to more sylvatic habitats of native species of the genus *Cochliomyia* and of resident *Lucilia eximia* (Guimarães et al*.,* 1978, Silva et al*.,* 2003). Extensive surveys for calliphorids carried out in Eastern (1971-72) and Southern (1974) Brazil (Guimarães et al*.,* 1978) did not report any specimen of *Chrysomya* spp. However, after their introduction they shortly became the predominant blowflies in Brazil accounting for 70% of the Calliphoridae captured in a recent survey in the Southern region, whereas *L. eximia* accounted for 0.75% and native *Cochliomyia* spp. for 1.2% (Batista-da-Silva et al., 2011).

Previous studies on trypanosomatids evidenced the association of *Herpetomonas* and *Angomonas* with flies and suggested that blowflies are common hosts for species of both genera (Teixeira et al*.*, 1997, 2011; Borghesan et al*.*, 2013). However, regarding the *Angomonas*/Calliphoridae association, the registers were too few (Teixeira et al*.,* 2011; Borghesan et

al., 2013; Týč et al., 2013) to permit a comprehensive analysis on the genetic diversity and bio-geographical structure of *Angomonas* populations. In addition, although the phylogenies of SHTs and respective TPEs were in general congruent corroborating co-divergent evolution, *A. deanei* and *A. ambiguus* shared the same symbionts. The partial congruence suggested both clonal propagation and recombination. The reduced genetic diversity within *Angomonas* composed by only three species harboring only two symbiont species could simply be due to small sample size. Although *Angomonas* spp. were reported parasitizing *Chrysomya* and *Lucilia*, the host and geographical ranges of this association were largely unknown. The lack of information on these subjects hampered the understanding of the role of both exotic and native blowflies in the origin, dispersion and species composition of *Angomonas.* Further works are required to know the composition of the trypanosomatid fauna of Neotropical and Afrotropical blowflies. Additional work was also necessary to know the genetic populational structure of *Angomonas* spp. and to better understand the co-divergence with their symbionts. The addressing of all these issues required wide-range phylogeographical analyses of SHTs of Neotropical and Afrotropical flies and demanded the use of molecular markers sensitive enough to assess the existence of both new species and cryptic intra-specific diversity of both, SHTs and TPEs. In this study, we propose to compare the tripartite partnership constituted by the trypanosomatid host, its proteobacterial endosymbiont and the flies that host the symbiotic aiming: to assess the genetic diversity of South American and African trypanosomatids from flies; to unveil the phylogeographical pattern and to hypothesize possible biogeographical scenarios for the origin and worldwide dispersion of the tripartite association, to evaluate co-divergent evolution of SHTs and their respective TPEs.

## Materials and Methods

### Areas of study, collection of flies, isolation and cultivation of trypanosomatids

Flies of several families have been captured from 1988 to 2013 in South America (Brazil, Venezuela and Colombia) and Africa (Guinea Bissau, Mozambique and Madagascar). In Eastern Africa, collections have been done at Mozambique in Maputo (district of Maputo) and Chupanga and Gorogonza (district of Sofala). In Western Africa collections were done at Guinea Bissau in the district of Buba (Reserve of Cufada). In Madagascar, collections were done in the Northern (Antsiranana), Central (Antananarivo) and Eastern regions (Moramanga and Andasibe). In Venezuela, the fly collections were done in Merida (1620m) and Laguna of Mucubaji at 3500m of altitude in the Cordillera de Merida (Andes Mountains) and in Santa Barbara at 130m of altitude (Province of Zulia). In Colombia, flies were collected in the Province of Tolima, at three sites of low altitude between the Central and Oriental Andes Cordilleras. In Brazil, flies were collected at several altitudes and biomes from North, West, Central and Southeast regions. The GPS and Table 1.

Table 1.Host and geographical origin of species of *Angomonas* and genotypes

| Trypanosomatid | TCC | Genotypes | order | family | Host origin species | Geographical origin City/State/country |
|---|---|---|---|---|---|---|
| ***A. desouzai*** | | | | | | |
| | | **Ads1** | | | | |
| | 079E | | D | Syrphidae | *Ornidia obesa* | Alfenas/MG/BR |
| | 1279 | | D | Calliphoridae | *Lucilia eximia* | Campo Grande/MS/BR (2006) |
| | 1310 - 12 | | D | Calliphoridae | *Lucilia eximia* | Campo Grande/MS/BR (2006) |
| | 1429 | | D | Calliphoridae | *Lucilia eximia* | Campo Grande/MS/BR (2007) |
| | 1453 | | D | Calliphoridae | *Lucilia eximia* | Campo Grande/MS/BR (2007) |
| | 2214 | | D | Calliphoridae | *Lucilia eximia* | Miranda/MS/BR (2011) |
| | 2459/ 60 | | D | Calliphoridae | *Crysomya* sp | São Carlos/SP/BR (2013) |
| | ISC0010a | | D | Calliphoridae | *Crysomya* sp | Mérida/Mda/Venezuela (2012) |
| | ISC0012a | | D | Calliphoridae | *Crysomya* sp | Potrerillo/Tol/CO (2013) |
| | ISC0016b | | D | Calliphoridae | *Crysomya* sp | Mérida/Mda/Venezuela (2012) |
| | ISC0001c | | D | Sarcophagidae | | Chupanga/Sofala/MZ (2012) |
| | ISC0022a | | D | Sarcophagidae | | Santa Barbara/Zulia/Venezuela (2012) |
| | | **Ads 2** | | | | |
| | 2317 | | D | Muscidae | *Musca* sp | Marromeu/Sofala/MZ (2012) |
| ***A. ambiguus*** | | | | | | |
| | 1780 | | D | Calliphoridae | *Chrysomya albiceps* | Campo Grande/MS/BR (2007) |
| | 1765 | | D | Calliphoridae | *Chrysomya putoria* | Buba/Tchingue/GB (2008) |
| | ISC0004a | | D | Calliphoridae | *Chrysomya putoria* | Antsitanana/MG (2012) |
| | ISC0010 | | D | Calliphoridae | *Crysomya* sp | Mérida/Mda/Venezuela (2012) |
| | ICS0011 | | D | Calliphoridae | *Crysomya* sp | Potrerillo/Tol/CO (2013) |
| | ISC0013b | | D | Calliphoridae | *Crysomya* sp | Ortega/Tol/CO (2013 |
| PNG-M02* | | | D | Calliphoridae | *Chrysomya megacephala* | Negada/Papua NG (2011) |
| ***A. deanei*** | | | | | | |
| Ke-19* | | | D | Sarcophagidae | *Wohlfahrtia nuda* | Todognang/Kenya (2009) |
| MCZ-01* | | | D | Calliphoridae | *Lucilia caesar* | Czechia (2010) |
| MCZ-12* | | | D | Calliphoridae | *Onesia austriaca* | Czechia (2011) |
| MMO-10* | | | D | Calliphoridae | *Acritochaeta orientalis* | Moranaga/MG |
| | | **Ade1** | | | | |
| | 036E | | H | Reduviidae | *Zelus leucogrammus* | Goiânia/GO/BR |
| | 263E | | D | Calliphoridae | *Chrysomya putoria* | Rolândia/PR/BR (1988) |
| | 080E | | D | Syrphidae | *Ornidia obesa* | Alfenas/MG/BR |
| | 1158 | | D | Syrphidae | *Ornidia obesa* | São Paulo/SP/BR (2006) |
| | 1503 - 05 | | D | Calliphoridae | *Chrysomya albiceps* | Campo Grande/MS/BR (2007) |
| | 1743* | | D | Calliphoridae | *Lucilia cuprina* | São Paulo/SP/BR (2008) |
| | 1752 | | D | Calliphoridae | *Lucilia cuprina* | São Paulo/SP/BR (2008) |
| | 1757 | | D | Calliphoridae | *Chrysomya putoria* | São Paulo/SP/BR (1989) |
| | 2315 | | D | Calliphoridae | *Chrysomya putoria* | Chupanga/Sofala/MZ (2012) |
| | 2314 | | D | Muscidae | *Musca sp* | Marromeu/Sofala/MZ (2012) |
| | 2316 | | D | Calliphoridae | *Chrysomya putoria* | Chupanga/Sofala/MZ (2012) |
| | ISC0001b | | D | Sarcophagidae | | Chupanga/Sofala/MZ (2012) |
| | | **Ade2** | | | | |
| | 1759 | | D | Calliphoridae | *Chrysomya putoria* | Buba/Tchingue/GB (2008) |
| | 2332 | | D | Muscidae | *Musca* sp | Marromeu/Sofala/MZ (2012) |
| | 2317 | | D | Muscidae | *Musca* sp | Marromeu/Sofala/MZ (2012) |
| | 2455 | | D | Muscidae | *Musca* sp | Ortega/TOL/CO (2013) |
| | | **Ade3** | | | | |
| | 1267 | | D | Calliphoridae | *Chrysomya putoria* | Maputo/MPM/MZ (2006) |
| | 1427 | | D | Calliphoridae | *Chrysomya albiceps* | Campo Grande/MS/BR (2007) |
| | 1445* | | D | Calliphoridae | *Chrysomya megacephala* | Campo Grande/MS/BR (2007) |
| | 1447 | | D | Calliphoridae | *Lucilia eximia* | Campo Grande/MS/BR (2007) |
| | 1639 | | D | Calliphoridae | *Chrysomya magacephala* | Chupanga/Sofala/MZ (2008) |
| | 1675 | | D | Calliphoridae | *Chrysomya putoria* | Maputo/MPM/MZ (2007) |
| | 1715 | | D | Calliphoridae | *Lucilia sp* | Bastos/SP/BR (1989) |
| | 1742 | | D | Calliphoridae | *Chrysomya megacephala* | Monte Negro/RO/BR (2008) |
| | 1754 | | D | Calliphoridae | *Chrysomya magacephala* | Monte Negro/RO/BR (2008) |
| | 1755/56 | | D | Calliphoridae | *Lucilia cuprina* | Monte Negro/RO/BR (2008) |
| | 1758 | | D | Calliphoridae | *Chrysomya magacephala* | Monte Negro/RO/BR (2008) |
| | 1760 | | D | Calliphoridae | *Chrysomya putoria* | Buba/Tchingue/GB (2008) |
| | 1762/63 | | D | Calliphoridae | *Chrysomya putoria* | Buba/Tchingue/GB (2008) |
| | 1884 | | D | Calliphoridae | *Chrysomya magacephala* | Confresa/MT/BR (2009) |
| | 1920 | | D | Sarcophagidae | | Gorongosa/ Sofala/MZ (2009) |
| | 1923 | | D | Calliphoridae | *Chrysomya putoria* | Chupanga/Sofala/MZ (2009) |
| | 1940 | | D | Calliphoridae | *Cochliomyia macellaria* | Miranda/MS/BR (2009) |

| | | | | |
|---|---|---|---|---|
| 2018 | D | Calliphoridae | *Chrysomya putoria* | Cufada/Buba/GB (2010) |
| 2025 | D | Calliphoridae | *Chrysomya putoria* | Cufada/Buba/GB (2010) |
| 2046 | D | Calliphoridae | *Chrysomya magacephala* | Campo Grande/MS/BR(2010) |
| 2052 | D | Calliphoridae | *Chrysomya magacephala* | Campo Grande/MS/BR (2010) |
| 2054 | D | Calliphoridae | *Chrysomya albiceps* | Campo Grande/MS/BR (2010) |
| 2065 | D | Calliphoridae | *Chrysomya magacephala* | Campo Grande/MS/BR (2010) |
| 2066 - 68 | D | Calliphoridae | *Chrysomya magacephala* | Campo Grande/MS/BR (2010) |
| 2070 | D | Calliphoridae | *Chrysomya albiceps* | Campo Grande/MS/BR (2010) |
| 2093 | D | Calliphoridae | *Chrysomya albiceps* | Nhecolandia/MS/BR (2010) |
| 2313 | D | Glossinidae | *Glossina* sp | Chupanga/Moçambique (2012) |
| 2454 | D | Sarcophagidae | | Araguaina/TO/BR (2013) |
| 2534 | D | Calliphoridae | *Chrysomya* sp | Moramanga/MG (2013) |
| 2554 | D | Muscidae | *Musca* sp | Moramanga/MG (2013) |
| 2601 | D | Calliphoridae | *Chrysomya* sp. | Moramanga/MG (2013) |
| ISC0004b | D | Calliphoridae | *Chrysomya putoria* | Antsitanana/MG (2012) |
| ISC0011a | D | Calliphoridae | *Chrysomya* sp. | Potrerillo/TOL/CO (2013) |
| ISC0013c | D | Calliphoridae | *Chrysomya* sp. | Ortega/TOL/CO (2013 |
| **Ade4** | | | | |
| 2446 - 50 | D | Muscidae | *Musca* sp | Payandé/TOL/CO (2013) |
| 2453 | D | Muscidae | *Musca* sp | Payandé/TOL/CO (2013) |

TCC (code number): Trypanosomatid Culture Collection, ISC: Insect Sample Collection
H, Hemiptera; D, Diptera
ISC (code number): Insect Sample Collection
BR, Brazil; Co, Colombia; VE, Venezuela; GB, Guinea Bissau; GT, MZ, Mozambique; MG, Madagascar, PNG, Papua New Guinea; SP, Sao Paulo; PR, Parana; GO, Goias; MG, Minas Gerais; MS, Mato Grosso do Sul; RO, Rondonia; MT, Mato Grosso; Mda, Merida; TOL, Tollima
*Sequences not determined in this paper

The intestinal contents of the insects were microscopically examined for flagellates. Smears on glass slides were prepared from the positive flies, fixed and stored. Positive gut samples were inoculated in culture medium and brought to our lab for clearing of contaminants and cultivation as described before (Sbravate et al., 1989). Flagellates obtained from cultures were cloned, and successively re-cloning to obtain pure cultures as determined by sequencing the PCR-amplified sequences without cloning. The established clean cultures were kept frozen in liquid nitrogen at the Trypanosomatid Culture Collection of the University of São Paulo, TCC-USP. Gut contents of microscopically positive insects were also preserved in 100% ethanol (v/v). In locations where microscopic examinations were unfeasible, the guts from dissected insects or entire insects were simply kept in ethanol. Culture and gut contents of flies before (Rodrigues et al., 2008).

**Selection of Symbiont Harboring Trypanosomatids (SHT) by DNA barcoding**

For the screening of SHT among the cultured flagellates or directly from the digestive tract of flies, DNA from these two sources were used for barcoding through V7V8 SSU rRNA sequencing as previously described (Teixeira et al., 2011; Borghesan et al., 2013). Those sharing high sequence similarity with *Angomonas* were submitted to further phylogenetic analysis.

**Phylogenetic analyses of the trypanosomatids (SHTs) based on gGAPDH sequences.**

The gGAPDH sequences from new SHT isolates selected by barcoding were amplified as described (Borghesan et al., 2013), and deposited in GenBank under the accession numbers listed in the Table 1. These sequences were aligned with corresponding sequences from all other SHT and from species of several genera representative of

the main clades in the Trypanosomatidae phylogenetic trees available in GenBank using ClustalX (Thompson et al., 1997), manually refined and submitted to phylogenetic inferences using parsimony (P), maximum likelihood (ML) and Bayesian inference (BI) methods. The parsimony and bootstrap analyses were performed using PAUP* version 4.0b10 software (Swofford, 2002), with 500 random sequence addition replicates followed by branch swapping (RAS-TBR). Bayesian analysis was performed using MrBayes v3.1.2 (Huelsenbeck et al., 2001). The tree searches employed GTR plus gamma and the proportion of invariable sites. The first 25% of the trees from 1,000,000 generations were discarded as burnin to allow the log-likelihood scores to reach stability. ML analyses were performed in RAxMLv.7.0.4 (Stamatakis, 2006) with 1,000 maximum parsimony starting trees. Bootstrap support was estimated with 1000 replicates in RAxML using the rapid bootstrapping algorithm (Stamatakis et al., 2008) and maximum parsimony as starting trees.

**Detection and phylogenetic analyses of the SHTs proteobacterial endosymbionts (TPE)**

Aiming to a specific, easy and rapid method for detecting and identifying TPEs in cultured trypanosomatids or directly from flies we developed a PCR assay based on polymorphic sequences of the GAPDH gene (~500bp) of TPEs. In addition, PCR was also standardized to amplify the Rpi (ribose 5-phosphate isomerase A) gene from TPEs (~550bp). To design the PCR primers, sequences from the genes of *A. deanei*, *A. desouzai* and *A. ambiguus* were obtained from the TPEs drafty genomes we recently have reported (Alves et al., 2013a). To amplify GAPDH sequences we employed the primers GAPDH.ENDO.F (5' AAG AGC TCA TTA TGA AGG TGG 3') and GAPDH.ENDO.R (5' TGG AAT CAT AYT CAT GGT TGC 3') in a reaction mixture (50ul) containing ~100 ng of DNA, 100 ng of each primer, 200 µM of each dNTP, 1.5 mM of $MgCl_2$ and 2.5 U of Taq DNA polymerase, under the following PCR conditions: 30 cycles with an initial cycle of 3 min at 94 $^{o}$C, 1 min at 94 $^{o}$C, 2 min at 58 $^{o}$C and 2 min at 72 $^{o}$C and a final cycle of 10 min at 72 $^{o}$C. The amplification of Rpi sequences were done using the primers RIB.ENDO.5F (5' GGR GTW GGW ACT GGA TCT AC 3') and RIB.ENDO.5R (5' ACC ACA AGT WAY AAC ACC TGG 3') using the same conditions employed for GAPDH but with a 60 $^{o}$C of annealing temperature. The amplification of ITS rDNA from TPE were performed as described previously (Teixeira et al., 2011). Phylogenetic analyses using sequences from GAPDH, Rpi and ITS rDNA were carried out using the methods reported above for SHTs phylogenetic inferences.

**Population genetic structure**

Correlation between geographic and genetic distance matrices was investigated employing the Mantel test version 2.0 (Liedloff, 1999), with 1,000 iterations for the calculation of the random distribution, Hypotheses of population genetic differentiation were tested by AMOVA, as implemented in Arlequin v. 3.5.1.2 (Excoffier et al.,

2005), with significance calculated using 20,000 permutations.

## Results

### Screening of SHTs in South American and African flies through DNA barcoding of cultured trypanosomatids

About 3700 flies of several families collected in South America (~84%) and Africa (~16%) were dissected and their fresh intestinal contents microscopically examined. About 1130 flies (~31%) resulted positive for trypanosomatids and the infection rates were similar for Neotropical and Afrotropical flies. Calliphoridae (~65% of the examined flies) followed by Muscidae (~31% of the flies examined) presented the highest prevalence of trypanosomatid infection. Within Calliphoridae, the species of *Chrysomya* and *Lucilia* were most commonly infected (~85% of the flies examined by microscopy) whereas lower infection rates (~20%) were detected in the New World native flies of *Cochlyomyia* from Brazil and Colombia.

The flagellates most commonly found in fly guts showed promastigote and choanomastigote forms displaying extremely varied morphology, suggestive of mixed infections. Flagellates resembling *Angomonas* in fresh preparations, i.e., small fast-moving choanomastigotes-shaped forms, were often observed in calliphorids, but their taxonomic positioning could not be ascertained by morphology alone. For this purpose, we compared the barcodes of 303 newly cultured isolates using the variable V7V8 region of SSU rRNA with those from 60 fly trypanosomatids already barcoded (Teixeira et al., 2011; Borghesan et al., 2013). The comparison of 364 cultures (~60% derived from calliphorids, 21% from Muscidae, 6% from Syrphidae and 4% from Sarcophagidae) showed that 70 (19%) corresponded to cultures *Angomonas* spp., mostly from calliphorids. Among the 75 cultures from Muscidae examined, only 12 (16%) were classified as *Angomonas*. Of 22 cultures from Sarcophagidae, only two was *Angomonas* while from 14 cultures from Syrphidae three were *Angomonas*. The new isolates of *Angomonas* could be classified into one of the three species of this genus: *A. deanei* (61 isolates), *A. desouzai* (16 isolates) and *A. ambiguus* (6 isolates) (Table 1; Fig.1).

### Survey of *Angomonas* directly in fly guts by PCR amplification of symbiont GAPDH sequences

Aiming to a better evaluation of *Angomonas* diversity, we selected gut samples from flies for a survey of *Angomonas* by two PCR approaches: V7V8 SSU rRNA and a specific PCR amplification of GAPDH sequences from TPEs. The last method revealed to be very effective and sensitive for specific detection of *Angomonas* in flies, and also for the selection of samples for further phylogenetic analyses. The subsequent barcoding of these samples permitted to detect flies simultaneously infected with the three species of *Angomonas* often associated with *Herpetomonas* spp. and flagellates of new taxa currently under characterization.

The PCR assay enhanced the detection of *Angomonas* compared to culturing; only ~32% of

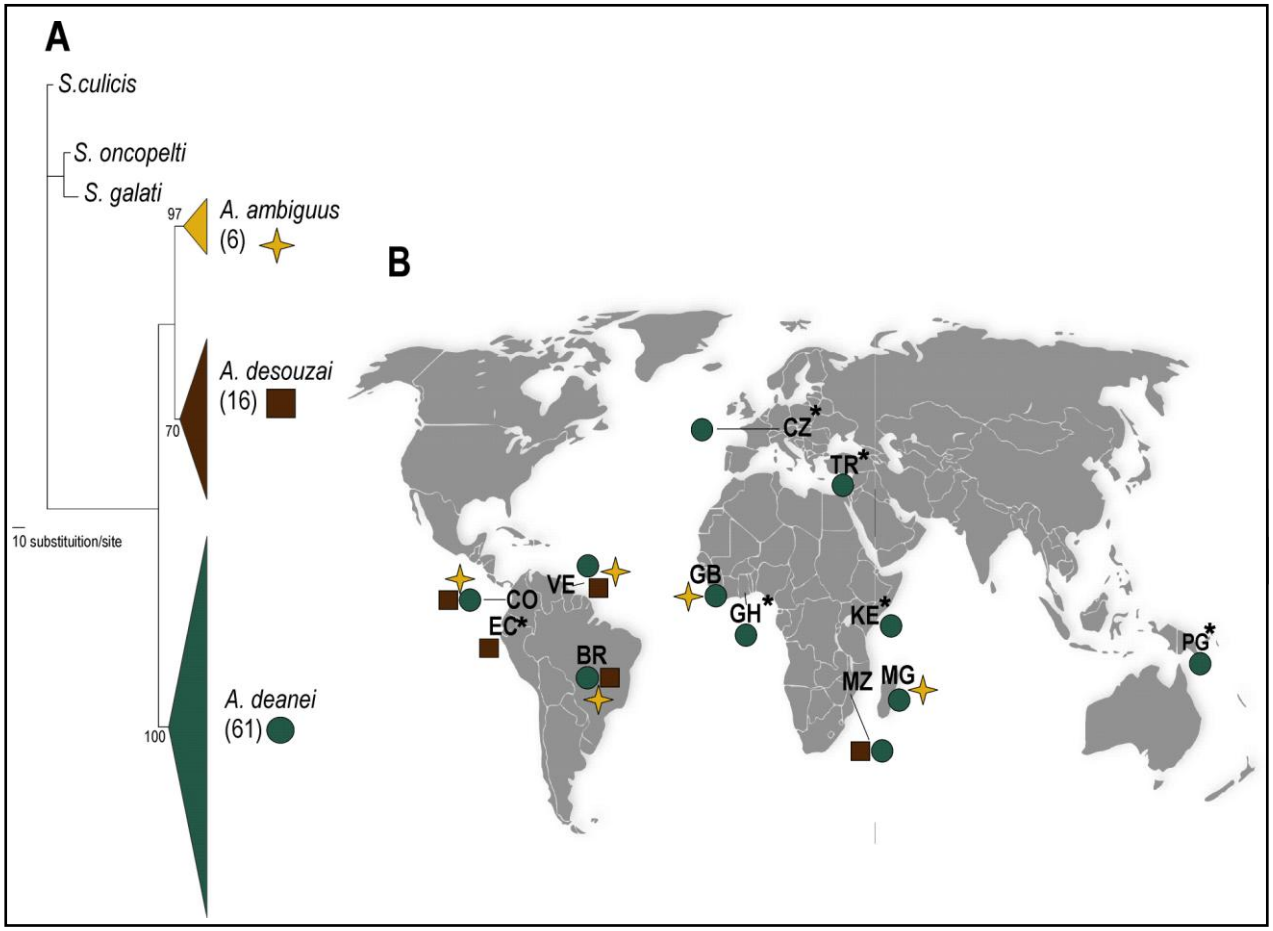


**Figure 1. (A)** Dendrogram inferred by parsimony analysis using V7V8 SSU rRNA sequences (769 characters, CI=087; RI= 0,87) from 82 isolates of *Angomonas* from flies. The number in parentheses indicates the total number of isolates positioned in each species. The numbers at nodes are bootstrap values derived from 100 replicates. (**B)** Map indicating the geographical origin of isolates of *Angomonas.* The (*) indicates the isolates obtained recently by Tŷč et al., 2013.

the trypanosomatid infected blowflies yielded cultures. In contrast, from 71 calliphorids collected in Africa, mostly *Chrysomya* spp., and in South America, mainly *Chrysomya* and *Lucilia,* 36 (51%) flies were positive for *Angomonas* in the PCR screening. Surveys for *Angomonas* directly in *Cochlyomyia* gut samples (from Brazil and Colombia) revealed 8 (~33%) positive flies among 24 infected by trypanosomatids.

**Genetic diversity and phylogenetic analyses of *Angomonas* inferred from gGAPDH sequences**

We selected *Angomonas* isolates from diverse hosts and geographic origins to include in phylogenetic analyses based on gGAPDH genes (Table 1). Phylogenetic trees inferred from concatenated SSU rRNA and gGAPDH trees (data not shown) corroborated the general branching pattern of the analyses restricted to gGAPDH. All phylogenetic trees, inferred by ML, MP and Bayesian methods, confirmed the monophyly of SHTs and its partition in two subclades corresponding to the genera *Strigomonas* and *Angomonas* (Fig. 2). The genus *Angomonas* included 73 new isolates plus 10 isolates obtained

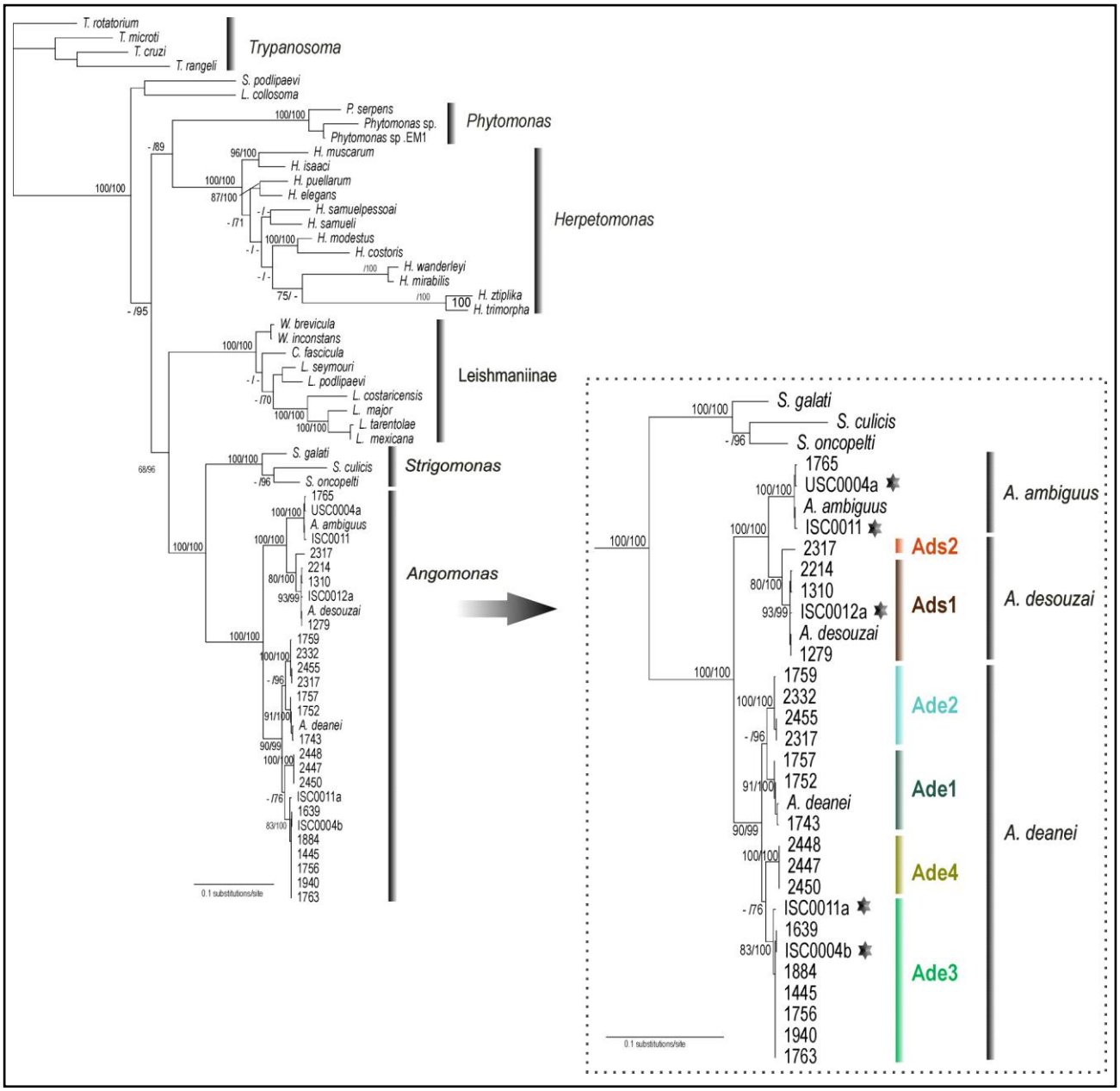


**Figure 2.** Phylogenetic tree of the symbiont harboring trypanosomatids (SHTs) and representatives of distinct trypanosomatid genera inferred by maximum likelihood (ML) analysis using concatened V7V8 SSU rRNA and gGAPDH gene sequences (alignment 1955 characters, Ln=-15649.515984). In the box dotted in featured are the subdivision of *Angomonas* in three major assemblages and their respective genotypes. The stars indicate isolates obtained directly from gut flies. The number in nodes refers to the bootstrap values derived from 500 replicates ML, BI and P.

in cultures and previously characterized (Teixeira et al., 2011) (Table 1). Species of *Strigomonas* were not detected in the flies examined. The analyses also confirmed the subdivision of *Angomonas* in three major assemblages corresponding to the three species, *A. deanei*, *A. desouzai* and *A. ambiguus* (Fig. 2), which were separated by gGAPDH sequence divergences ranging from 4% between *A. desouzai* and *A. ambiguus* to 7% between *A. ambiguus* and *A. deanei* (Table 2).

The analyses of gGAPDH sequences from a large number of flagellates from cultures (70) or directly from flies (13) disclosed a significant cryptic diversity within *A. deanei* and *A. desouzai* undetected by the more conserved SSU rRNA sequence analyses. The gGAPDH sequence

Table 2. Nucleotide divergence (%) of genus *Angomonas* and their respective genotypes

| Genotypes of *Angomonas* | Ade1 | Ade2 | Ade3 | Ade4 | Ads1 | Ads2 |
|---|---|---|---|---|---|---|
| **gGAPDH** | | | | | | |
| Ade1 | - | | | | | |
| Ade2 | 2 | - | | | | |
| Ade3 | 3 | 3 | - | | | |
| Ade4 | 3 | 3 | 2 | - | | |
| Ads1 | 7 | 7 | 7 | 7 | - | |
| Ads2 | 7 | 7 | 7 | 7 | 2 | - |
| **Genotypes of endosymbiots** | **SAde1** | **SAde2** | **SAde3** | **SAde4** | **SAds1** | **SAds2** |
| **GAPDH** | | | | | | |
| SAde1 | - | | | | | |
| SAde2 | 2,5 | - | | | | |
| SAde3 | 8 | 9 | - | | | |
| SAde4 | 8 | 9 | 1,5 | - | | |
| SAds1 | 23 | 24 | 24 | 25 | - | |
| SAds2 | 23 | 24 | 24 | 25 | 9 | - |

divergences separating the subclades within *A. deanei* ranged from ~2 to 3% (Table 2). Actually, the species *A. deanei* comprises four genotypes and *A. desouzai* two genotypes. The *A. deanei* genotypes will be hereafter designated Ade1, Ade2, Ade3 and Ade4. The commonest genotypes within the clade *A. deanei* were Ade3 (62%), followed by Ade1 (21%), Ade4 (10%) and Ade2 (6.5%). Ade1 comprises the reference isolate of this species (Fig. 2, Fig. 3). Despite the smaller number of isolates, relevant polymorphisms were detected within the clade *A. desouzai.* Sequence divergence of 2% in gGAPDH permitted to define two genotypes: Ads1 comprising the reference species *A. desouzai* plus 9 sequences from three cultured and 5 uncultured isolates, and Ads2 with only one isolate (TCC2317) from Mozambique, which was assigned to Ads2 and diverged ~2% from the reference *A. desouzai* of the genotype Ads1 (Fig. 3, Table 2). So far, the 6 isolates of *A. ambiguus* examined (2 from cultures and 4 directly from fly guts) showed more homogenous sequences (~0.5% divergence) compared to the other two species.

**Phylogenetic relationships among the symbionts of *Angomonas* and congruence analyses of host and symbiont phylogenies**

Phylogenetic analyzes of variable sequences from GAPDH, Rpi and ITS DNA sequences from representatives of all genotypes of the three *Angomonas* species, confirmed previous results of Teixeira et al. (2011) showing that the symbionts from *Angomonas* belong to two distinct "*Candidatus*" species of Betaproteobacteria: "*Ca*. Kinetoplastibacterium desouzaii", hosted by *A. desouzai,* and "*Ca.* K. crithidii" hosted by *A. deanei* and *A. ambiguus.* In consonance with the phylogeny of theirs hosts, the phylogenetic analyzes of the endosymbiont showed that *A. desouzai* harbors two distinct symbiont subclades while *A. deanei* harbors four symbiont subclades. The four TPEs genotypes (subclades) within the clade "*Ca*. K. crithidii" were called SAde1-SAde4. TPE genotypes within the clade "*Ca. K.* desouzai" were called SAds1 and Sad2. The symbionts of all isolates of *A. ambiguus* clustered within the subclade SAd3 of "*Ca*. K. crithidii". Divergences among the GAPDH sequences from the symbionts

of the *Angomonas* genotypes ranged from 2.5 to 9.0% within "*Ca*. K. crithidii" and from 1 to 9% within "*Ca*. K. desouzai" (Table 2). Sequences from TPEs obtained directly from gut flies clustered with those from genotypes of TPEs described for cultured isolates of *Angomonas* (Fig. 3).

The congruence between host and symbiont phylogenetic trees revealed by Tree Map analysis for most isolates of *Angomonas* included in this analysis strongly indicated co-divergence of the genotypes of *A. deanei* and *A. desouzai* with their respective symbionts. The results also confirmed the lack of congruence between *A. ambiguus* isolates and their symbionts (Fig. 4). SAd1 was exclusively associated with Ade1, SAd2 with Ade2, SAd3 with Ade3 and Sad4 with Ade4. However, in one isolate of *A. deanei* parity was not observed; this isolate exhibited host of Ade3 genotype while its symbiont was SAde4. Similarly, Ads1 and Ads2 were respectively associated with SAds1 and SAds2. These findings exhibit some parallelism withthe genetic ambiguity of *A. ambiguus*, which is phylogenetically closest to *A. desouzai,* but whose symbiont is closest to the symbiont of *A. deanei* (Teixeira et al., 2011). This observation, suggestive of hydridization, was initially based on one Brazilian isolates of *A. ambiguus*. The inclusion in the present study of three sequences from symbionts of two new isolates of *A. ambiguus* from culture and directly from flies confirmed that the symbiont of *A. ambiguus* is indeed "*Ca.* K. crithidii" and, in addition, demonstrated that all TPEs from this species nested into the SAd3 genotype of "*Ca.* K. crithidii" (Fig. 3).

**Association between *Angomonas* species/genotypes and host flies**

Together, cultured and non-cultured samples accounted for a total of 83 *Angomonas* isolates from blowflies characterized in this study. Within Calliphoridae, the species of *Chrysomya* and *Lucilia* were the main hosts of the *Angomonas* showing 74% of cultures and ~51% of flies positive for *Angomonas* by PCR, whereas one out of 19 cultures from native calliphorids was *Angomonas* and 33% of flies tested by PCR was positive for this genus. Here, 52 out of 225 cultures (~23%) from calliphorids were classified as *Angomonas.* The remaining cultures were classified as *Herpetomonas* or belong to new taxa currently under characterization. The infections rates of *Angomonas* among the 75 cultures from Muscidae (16%), the 22 cultures of Sarcophagidae (9%) and 14 from the Syrphidae (21%) were significantly lower than in Calliphoridae. The *Angomonas* isolates detected in these flies were all assigned to two one (Ade1 and Ade2) of the four genotypes of *A. deanei.* Many isolates from these two last fly families were *Herpetomonas,* which is also a genus commonly hosted by blowflies (Borghesan et al., 2013 and unpublished data) (Table 1).

It is important to note that the number of *Angomonas* cultures recovered does not express the actual prevalence of these parasites in flies. There are many operational factors against a successful cultivation of flagellates from insects,

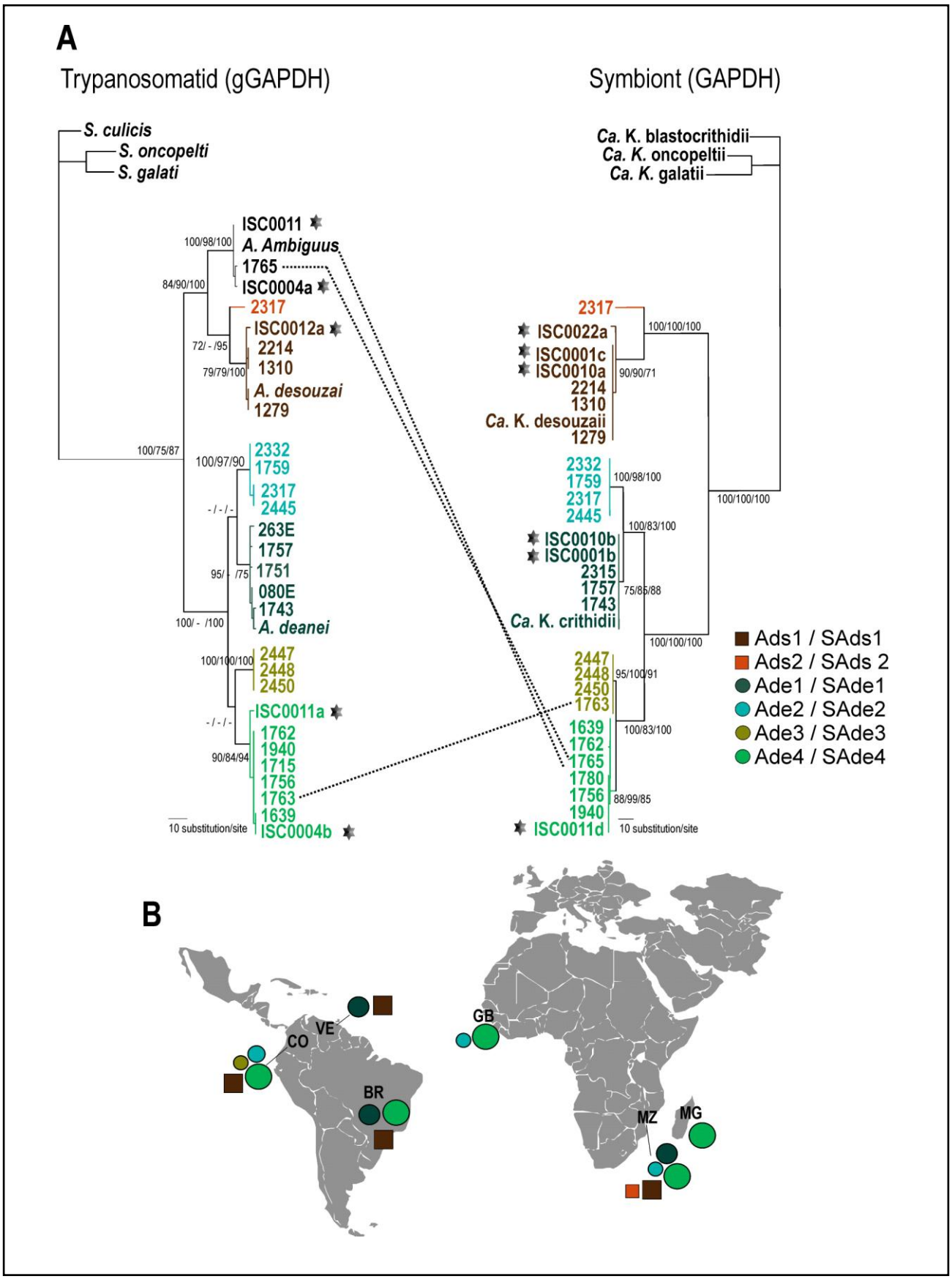


**Figure 3. (A)**. Parity analysis of phylogenetic trees (parsimony analyses) of gGAPDH sequences from the trypanosomatids (864 characters, CI=0,7, RI= 0,9) and GAPDH from the symbionts (502 characters, CI=0,8, RI= 0,9). The stars indicate isolates obtained directly from gut flies. The dotted lines indicate no parity of *A. ambiguus* and *A. deanei* isolates with their symbionts. The numbers in the nodes correspond to boostrap values derived from 100 replicates for P, ML and BI. **(B)** The map indicates the geographical origin of the *Angomonas* genotypes.

with the strong contamination by bacteria and fungi being the main limiting factor, particularly in the absence of lab facilities near to collection sites. Nevertheless, we obtained 225 cultures from calliphorids, 52 (~23%) classified as *Angomonas.* Thus, although sub-estimated, available data from this and previous studies (Borghesan et al., 2013) indicate that *Angomonas* are extremely common in blowflies.

The species and genotypes of *Angomonas* did not show any strict preference for determined species or genus of blowflies (Table 1). All the new isolates of *Angomonas,* regardless their African or South American origin, could be classified in one of the three species of this genus. The 13 uncultured isolates characterized by sequencing analysis (based on the host trypanosomatid gGAPDH and V7V8 SSU rRNA) from the guts of flies positive for *Angomonas* by PCR amplification of the symbiont GAPDH gene were identified as *A. deanei* (31%), *A. desouzai* (38%) and *A. ambiguus* (31%).

**Lack of geographical structure of *Angomonas* species and genotypes**

Regarding the geographic origin of the 70 *Angomonas* cultures, 41 isolates were from Brazil, 7 from Colombia, 7 from Guinea Bissau, 12 from Mozambique and 3 from Madagascar. This preponderance of Brazilian isolates most likely reflects the better laboratory facilities and technical support we had in Brazil.

Taken together, data from cultures and surveys directly from fly guts revealed wide distribution of all *Angomonas* species and genotypes. The three species of *Angomonas* are present in South America and Africa*: A. deanei* (~81% of *Angomonas* cultures and ~31% of the trypanosomatids detected directly on fly guts) is the most prevalent species and ubiquitous to the 6 countries included in this study. Recently, it was reported in flies from Ghana, Kenya, Turkey, Czech and Papua New Guinea (Týč et al., 2013). *A. desouzai* (16%, culture and 38%, fly guts) and *A. ambiguus* (3% and 31%) were also found in most regions investigated in this study. *A. desouzai* was found in Brazil, Venezuela, Colombia and Mozambique, and *A. ambiguus* in Brazil, Venezuela, Colombia, Guinea Bissau and Madagascar. *A. desouzai* was also reported from Equador (South America) and *A. ambiguus* from Papua New Guinea (Týč et al., 2013) (Fig. 1, Table 1).

*Angomonas* spp. could be recovered from calliphorids of the most diverse habitats, from both hemispheres, from zero to 3.500m of altitude in the Andes Cordillera, from 8º North (Venezuela) to 25º South (South Brazil) and from 47º East (Madagascar) to 76º West (Colombia). In Brazil, *Angomonas* occurred in flies from all biomes, including the Amazonian Rainforest, the Pantanal wetlands and the arid Caatinga (Fig. 1, Table 1). Therefore, *A. deanei* genotypes dispersed randomly through the Neotropic and Afrotropic. No correlation was found regarding geographic and genetic distances between isolates of most genotypes of *A. deanei*.

**The taxonomical status of *A. deanei* and *A. desouzai* genotypes**

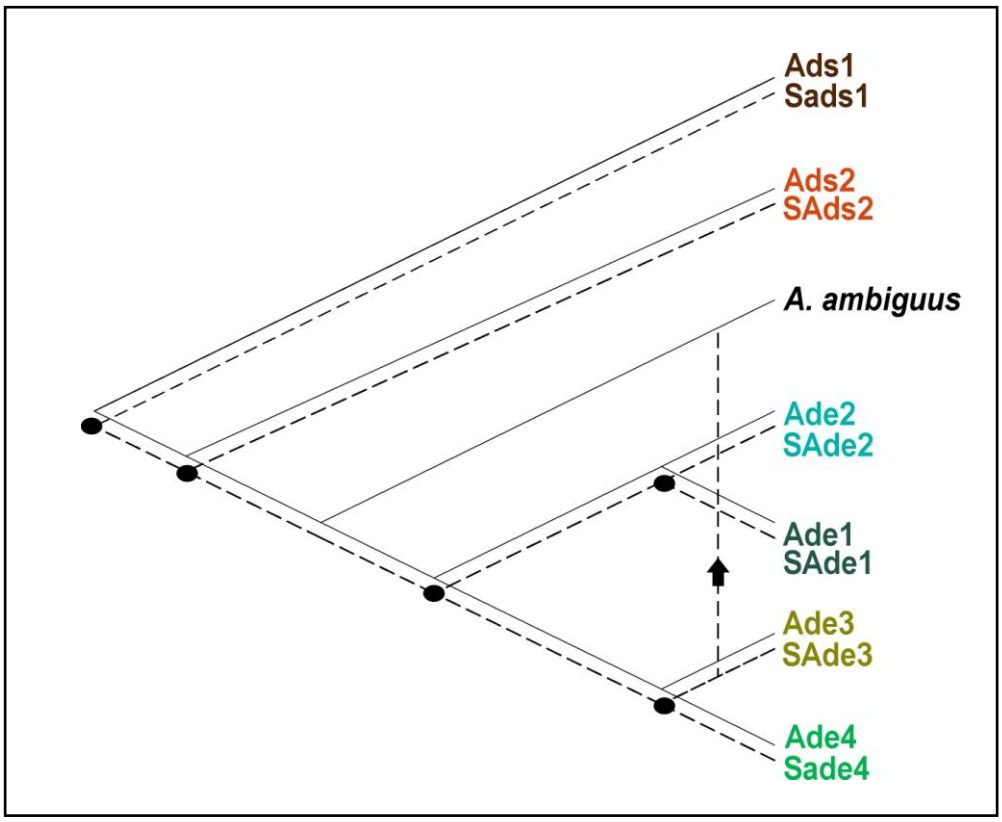


**Figure 4.** Reconstruction of the Tree Map analysis showing co-divergence (●) of the genotypes of *A. deanei* and *A. desouzai* and respective genotypes of symbionts. The solid line is the reconstruction of the trypanosomatids hosts and the dashed line are the symbionts. The arrow indicates no congruence between *A. ambiguus* and their symbiont.

The gGAPDH sequence divergences separating the subclades within *A. deanei* ranged from ~2 to 3%; the largest divergence separated *A. deanei* Ade1 from Ade3 and Ade4 genotypes. Within the clade *A. desouzai*, divergences ranged from 1 to 2%, with the largest distances separating Ads1 and Ads2. Regarding GAPDH from the symbionts, divergences ranged from 2,5 to 9% within the clade *A. deanei* and 1 to 9% in *A. desouzai* (Table 2). Although the GAPDH divergences among the genotypes within each species are three times smaller than the divergences between the three known species of *Angomonas,* which stand around 7-8% for the SHTs and 23-25% for their symbionts, the divergences together with the host-symbiont association permitted to consider the herein named genotypes Ade3-Ade4 and Ads2 as two new species. In this study, we opted to provisionally consider them just as genotypes of *Angomonas* until additional morphological data and additional molecular data are gathered.

## Discussion

In this paper we evaluated the genetic diversity and the biogeography of trypanosomatids parasitizing dipterans of South America and Africa and particularly compared the diversity of SHTs and TPEs genotypes of the Neotropic with those genotypes recently introduced into the Neotropic by blowflies of African origin. The preferred hosts of *Angomonas* are dipterans and among them the cosmopolitan species of Calliphoridae. In order to

compare the *Angomonas* faunae of the Neotropical and Afrotropical ecozones, we analyzed 62 isolates from calliphorids from South America (Brazil, Colombia and Venezuela) and Africa (Guinea Bissau, Mozambique and Madagascar) by barcoding through V7V8 SSU rRNA sequencing. Among 364 cultures isolated from flies, 23% were identified as *Angomonas*. In addition to cultured organisms, 13 non-cultured isolates from flies infected by *Angomonas* (PCR assay targeting the symbionts) were identified by sequencing analysis of PCR-amplified DNA from the host SHTs and their symbionts.

Despite the large amount of cultures and the surveys for species directly on fly gut contents by PCR, the species composition of *Angomonas* proved to be reduced to three species. Nevertheless, the results from direct fly screening showed that *A. desouzai* and *A. ambiguus* are more common in blowflies than culturing suggested, what is suggestive of culture selection favoring *A. deanei*. In fact, the species of *Angomonas* spp. differs in growth behavior and *A. ambiguus* is the most fastidious species of this genus (unpublished data). Therefore, the possibility that the species richness could become higher when flies were directly probed for SHTs was not confirmed. In addition, the expected accumulation of species and larger parasite diversity in areas of parasite introduction was not supported by the analyses of this study: only *A. deanei, A. ambiguus* and *A. desouzai* occurred in South American as well as in African flies.

The comparison of the isolates of *Angomonas* through phylogenetic analysis disclosed relevant cryptic diversity distinguishing intra-specific genotypes. The phylogenies of the SHTs proteobacterial endosymbionts (TPEs) based on gGAPDH, RPI and ITS rDNA were totally congruent, and showed highly concordance with the gGAPDH-based phylogeny of their hosts. The four genotypes of *A. deanei* were congruent with the four genotypes of "*Ca. K. Crithidii*" while the two genotypes of *A. desouzai* were also congruent with the two genotypes of "*Ca. K. desouzai*", thus corroborating the SHT-TPE co-divergence and their shared evolutionary histories. Nevertheless, in addition to all isolates of *A. ambiguus* that share symbiont with *A. deanei* at least one isolate of each *A. deanei* and *A. desouzai* revealed incongruent SHT and TPEs phylogenies, which is suggestive of recombination resulting in hybrid genotypes. Although the cultures have been cloned twice and checked regarding clonality by direct sequencing of SSU rRNA and gGAPDH amplified sequences, it cannot be absolutely ruled out that we are dealing with mixed cultures composed by more than one genotype and are artificially amplifying gene from distinct organisms. We cannot state that these isolates are indeed hybrid lineages until undergoing and more conclusive molecular analyses are completed.

Blowflies of the genera *Chrysomya* and *Lucilia* hosted the majority of the *Angomonas* identified. These blowflies not only harbored more than one species and genotype of *Angomonas*, but also harbored, simultaneously or not, other trypanosomatid genera, particularly *Herpetomonas,* and new trypanosomatid taxa not assignable to any known genus (unpublished data). This and previous

studies (Teixeira et al., 2011; Borghesan et al., 2013) indicate that *Angomonas* and *Herpetomonas* are quite common in blowflies. In a previous study, we demonstrated that some species of *Herpetomonas* are generalist parasites of flies and appear to be as cosmopolitan as their hosts. Of the 52 flagellates assigned to the genus *Herpetomonas* 90% were recovered from flies of Calliphoridae, 51%; Muscidae, 21%; Syrphidae, 11%; Sarcophagidae, 9% and Ceratopogonidae (4%) (Borghesan et al., 2013).

Calliphorids of *Chrysomya* and *Lucilia* seem to be the principal hosts of *Angomonas* both in the Neotropical and Afrotropical ecozones, whereas low infection rates by *Angomonas* was detected in the native South American calliphorids of *Cochliomyia.* The low prevalence in flies of other families and the absence of *Angomonas* in non-predator hemipterans add additional support to *Angomonas* preference for blowflies. However, despite the strong association of *Angomonas* with blowflies, other dipteran families can also host *Angomonas* spp. These are the cases of the Muscidae (mostly domestic fly) and Sarcophagidae, which share anthropogenic niches and alimentary habits with the Calliphoridae, and of the Syrphidae, which like blowflies also fed on fruits and nectar. The sharing of niches and the fact that transmission of trypanosomatids among flies occurs by oral contamination with feces probably account for the presence of *Angomonas* in blowflies, domestic flies, sarcophagids and syrphids.

The identical species composition in distant geographic sites and distinct ecological niches and the worldwide dispersion of *Angomonas* spp. are compatible with the recent and widespread blowfly dispersion. *Angomonas* occurs in blowflies from the Andean mountains to the seaside of South America and Africa, in blowflies from the rainforest and wetlands, and from the savanna and other arid and semi-arid biomes. There is no correlation between the genetic diversity and geographic distances of the genotypes of *Angomonas*. The three species of *Angomonas* appear to disperse ubiquitously through the Neotropics and Afrotropics failing to show any spatial structure at species and genotype levels. Anthropogenic action certainly favors the widespread dispersal of *Angomonas*-infected blowflies.

The finding of opisthomorphs typical of SHTs in early drawings of trypanosomatids from African blowflies indicates that the presence of SHTs in these hosts is at least two centuries old (Roubaud, 1908, 1911a,b). This fact might suggest that blowflies could be responsible for the introduction of SHTs into the Neotropics sometime before the recent *Chrysomya* invasion. Calliphorids of the genus *Lucilia* have been reported in Brazil since the years 1930 (in Mello, 1961) while African blowflies of *Chrysomya* were introduced to Brazil and to the Neotropics very recently (1976), but before that *A. deanei* was already isolated from a predator hemipteran in Brazil (Carvalho et al., 1973). In fact, in the last five centuries calliphorids had ample opportunities to carry *Angomonas* from West Africa and Europe (Iberian Peninsula) to South America and vice-versa due to the intense intercontinental trade of livestock, goods and slaves. Actually, the first shipments of cattle came to Brazil at 1534 from the Açores and at 1549 from

Cabo Verde (Bueno, 2002; Mariante et al., 2006), and the slave traffic from Africa was intense from the XVI$^{th}$ to the XIX$^{th}$ centuries (Thorton, 1998). At present, the introduced *Chrysomya* spp. are the most prevalent blowflies in the Neotropics.

The results from this study suggests that the historical dispersion and colonization of blowflies around the word, together with host switching between introduced and native flies, mostly within calliphorids and at small degrees to other dipteran families sharing ecological niches, shaped the diversity and distribution of SHT species. In the absence of paleontological landmarks we cannot precisely establish the geological time of the colonization of calliphorids by *Angomonas*. However, some considerations can be raised about the events leading to the tripartite association of calliphorid-*Angomonas*-symbiont. The origin of the Calliphoridae subfamilies Lucilinae and Calliphorinae got distant timing estimates, from the late Cretaceous to the early Miocene. *Chrysomya* and *Cochliomyia* are members of Calliphorinae originally from the Old and New Worlds, respectively, suggesting that their ancestors were separated long time ago (Gaunt & Miles, 2002; Stevens & Wallman, 2006; Wallman et al*.,* 2005). There are fossil records showing trypanosomatids associated to insects since the early Cretaceous (Poinar Jr, 2008), but no fossil records exist of blowfly infected by trypanosomatid.

The time of colonization of *Angomonas* by its symbionts is also unknown, but it is currently well established that all TPEs, from *Angomonas* and *Strigomonas*, have a common betaproteobacterial ancestor closest to *Taylorella* spp*.* of the family Alcaligenaceae (Alves et al., 2011; 2013 a,b; Motta et al., 2013). The common origin of all TPEs suggests that the colonization of SHTs resulted from the infection with a single proteobacterial species. This possibility is strongly supported by the overall genomic similarity between the symbionts of all *Angomonas* and by the synteny of their genes (Alves et al*.,* 2013a). It has also been suggested that the infection of all SHTs by a proteobacterial species occurred only once as a single event (Du et al., 1994a, Teixeira et al*.,* 2011). Du et al. (1994b) calculated this event to have occurred ~120 mya, at mid Cretaceous, based on 16S divergences between "*Ca*. K. crithidii" and a *Bordetella* ancestor, using as reference the mutational rate of aphid symbionts (Moran et al., 1993). Applying the same calculation process to a *Taylorella* ancestor, the time of the colonization of *Angomonas* by TPEs got estimates of similar magnitude. These estimates imply that the infection of *Angomonas* by the symbionts at mid Cretaceous predated the geological time of the origin of calliphorids. Therefore, the extant calliphorids would have acquired their SHTs either from an ancestor or from an insect of a distinct taxon. Comparison of the sequence divergences between the extant symbionts and their divergences from their putative *Tayrorella* ancestor allows hypothesizing that the split between the extant symbiont species is three to four times more recent than the colonization event (at the late Cretaceous ~120 MYA). This would place the splitting between the symbionts of *A. deanei* and *A. desouzai* somewhere at the Eocene/Oligocene; by that time calliphorids were

already available as hosts (Gaunt & Miles, 2002; Stevens & Wallman, 2006; Wallman et al., 2005).

The pairs of host/symbiont genotypes closely knitted as evidenced by congruent host and symbiont phylogenies are a contemporary example of codiversification by coevolution in the sense that the host-symbiont pairs are diversifying together within a shared history of millions of years of interplay between host and symbiont genomes and vertical descent (Moran, 2006; Koreny et al., 2010; Alves et al., 2011, 2013a). It deserves emphasizing that, in general, the diversifying subpopulations of *Angomonas* do not exchange symbionts, even if they share the same host species, and *Angomonas* spp. behave as asexual, clonal populations that usually do not recombine. The predominance of clonal propagation reflects the paucity of putative hybrids such as those represented by *A. ambiguus* that may be regarded as a hybrid lineage as well as a few isolates of *A. deanei.*

Data from the analyses of STHs or TPEs indicated that all species and, apparently, all genotypes of *Angomonas* are cosmopolitan. In fact, cosmopolitanism appear to be a common feature of insect trypanosomatid as demonstrated before for some species of *Leptomonas, Herpetomonas* and *Angomonas* (Votýpka et al., 2009, 2012; Teixeira et al., 2011; Týč et al., 2013). The findings of identical or highly similar genetic repertoire of *Angomonas* in distant locations from the New and Old Word indicated that their separation is quite recent, consistent with the historical worldwide dispersion of blowflies. This study shows that we are witnessing an explosive radiation of the pairs of trypanosomatid-symbiont, whose universal dissemination is guaranteed by a third party, their highly vagile and cosmopolitan hosts, the Calliphoridae.

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ORIGINAL PAPER

# Molecular Phylogenetic Redefinition of *Herpetomonas* (Kinetoplastea, Trypanosomatidae), a Genus of Insect Parasites Associated with Flies

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**In order to review the taxonomy of the genus *Herpetomonas* through phylogenetic and morphological analyses we barcoded 527 insect trypanosomatids by sequencing the V7V8 region of the small subunit ribosomal RNA (SSU rRNA) gene. Fifty two flagellates, 90% of them from Diptera, revealed to be related to known species of *Herpetomonas*. Sequences of entire glycosomal glyceraldehyde phosphate dehydrogenase (gGAPDH) and SSU rRNA genes were employed for phylogenetic inferences including representatives of all genera of Trypanosomatidae. In the resulting phylogenetic trees, the selected flagellates clustered into a monophyletic assemblage that we are considering as the redefined genus *Herpetomonas*. Internal transcribed spacer 1 (ITS1) rDNA sequences and putative secondary structures of this region were compared for evaluation of inter- and intraspecific variability. The flagellates were classified in six already known species and five new species. In addition, two *Leptomonas* spp. were moved to *Herpetomonas*, now comprising 13 valid species, while four species were excluded from the genus. Light and electron microscopy revealed the extreme polymorphism of *Herpetomonas*, hindering genus and species identification by morphological characteristics. Our findings also showed that some species of *Herpetomonas* are generalist parasites of flies and appear to be as cosmopolitan as their hosts.**


**Key words:** Diptera; insect trypanosomatids; *Herpetomonas*; morphology; phylogeny; taxonomy.

## Introduction

Flagellates of the family Trypanosomatidae constitute a monophyletic set of obligate parasites of vertebrates (genera *Trypanosoma, Leishmania* and *Endotrypanum*) and plants (*Phytomonas*) as well as several genera of parasites of invertebrates, mostly insects. The trypanosomatids of insects were originally classified by morphology, host origin and life cycle and accordingly placed by Hoare and Wallace (1966) in the following four genera: *Blastocrithidia*, *Crithidia*, *Leptomonas* and *Herpetomonas*. In addition to these genera, various authors have created new genera for insect trypanosomatids, including *Cercoplasma* (Roubaud 1911a), *Rhynchoidomonas* (Patton 1910), *Cystotrypanosoma* (Roubaud 1911b), *Malacozoomonas* (Nicoli et al.

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1971a), *Nematodomonas* (Nicoli et al. 1971b), *Wallaceina* (Merzlyak et al. 2001; Podlipaev 1990), *Strigomonas* (Lwoff and Lwoff 1931; Teixeira et al. 2011), *Angomonas* (Teixeira et al. 2011) and *Sergeia* (Svobodová et al. 2007). Only the last four of these proposed genera are currently in use and all were phylogenetically validated.

The genus *Herpetomonas* was created to accommodate flagellates showing pro- and opisthomastigote forms in their life cycles (Hoare and Wallace 1966). In the promastigotes, the kinetoplast is anterior to the nucleus while in the opisthomastigotes it is posteriorly located. In principle, the placement of a new trypanosomatid in this genus should be eased by the presence of opisthomastigotes in their host insects and/or cultures. However, opisthomastigotes can be rare or absent in insects and cultures, and they also can be confounded with flagellates showing posteriorly located kinetoplasts, similar to the opisthomorphs, which are choanomastigote-shaped multiplying forms with the kinetoplast posterior to the nucleus, of the genera *Angomonas* and *Strigomonas* (Teixeira et al. 1997, 2011). These uncertainties have been a source of classification mistakes leading to the placement of *Herpetomonas* within other promastigote-displaying genera (*Leptomonas* and *Phytomonas*), or inversely, classifying as *Herpetomonas* flagellates now ascribed to different genera (Teixeira et al. 1997, 2011).

In general, *Herpetomonas* thrive in dipterans (Teixeira et al. 1997; Wallace 1966). They have also been reported, either correctly or incorrectly, in predator hemipterans (Roitman et al. 1976), plants (Fiorini et al. 2001), an Egyptian rat (Podlipaev et al. 2004b) and even in immunodepressed humans (Chicharro and Alvar 2003; Morio et al. 2008). In their more common hosts, the Diptera, promastigotes live in the digestive tract, preferentially in the rectum, attached either to endothelian cells or as free-swimmers among fewer opisthomastigotes (Hupperich et al. 1992; Nayduch 2009). Transmission among flies and hemipterans is thought to occur respectively through coprophagy and predation/cannibalism.

The description of a new species of *Herpetomonas* was usually based on its morphology and host origin. These criteria supported the description of more than a hundred *Herpetomonas* spp. (Podlipaev 1990; Wallace 1966; Wallace et al. 1983). Attempts to distinguish species of *Herpetomonas* using molecular markers were initially based on restriction site polymorphisms and cross-hybridization using ribosomal DNA and kDNA sequences (Camargo et al. 1992; Nunes et al. 1994; Teixeira et al. 1997). These markers distinguished species of *Herpetomonas* and disclosed species that, although formerly classified in this genus, were more related to other trypanosomatid genera. Randomly amplified polymorphic DNA (RAPD) patterns in addition to restriction fragment length polymorphism (RFLP) analyses of PCR-amplified SSU rRNA and ITS rDNA revealed species and intra-specific polymorphisms within *Herpetomonas* (Fiorini et al. 2001). Although available from a few species, sequences from SL and 5S rRNA proved to be useful markers for distinguishing organisms within this genus (Podlipaev et al. 2004b) as demonstrated for insect trypanosomatids of other genera (Fernandes et al. 1997; Maslov et al. 2007, 2010; Serrano et al. 1999; Teixeira et al. 1996; Westenberger et al. 2004). Recently, a combination of phylogenetic and morphological data were used in the description of new species of *Herpetomonas* (Podlipaev et al. 2004a; Zídková et al. 2010).

Several studies have shown that morphology can no longer be held as the sole criteria for the classification of trypanosomatid genera and species while molecular data provide invaluable support for the taxonomy of insect trypanosomatids (Camargo et al. 1992; Fernandes et al. 1997; Maslov et al. 2007; Merzlyak et al. 2001; Nunes et al. 1994; Podlipaev et al. 2004a, b; Teixeira et al. 1996, 1997, 2011; Westenberger et al. 2004; Yurchenko et al. 2006a, b, 2008; Zídková et al. 2010). These studies raised the expectation that the use of ambiguity-free molecular characters could produce a reliable and less subjective taxonomy. However, it soon became apparent that the reliability of molecular data could be hampered by samples that are too small and/or by samples spoiled by misclassified organisms. For instance, the genus *Herpetomonas* was thought to be polyphyletic because, in some phylogenetic trees, flagellates classified as *Herpetomonas* according to the morphological criteria were positioned within other trypanosomatid genera (Hughes and Piontkivska 2003; Maslov et al. 2010; Podlipaev et al. 2004a; Svobodová et al. 2007; Zídková et al. 2010).

In this study, a large number of *Herpetomonas* spp. was positioned in the trypanosomatid phylogenetic tree and the relationships among the species within this genus were inferred. With this purpose, we assessed the genetic diversity of new trypanosomatids from insects by DNA barcoding and inferred phylogenetic trees using SSU rRNA and gGAPDH genes from the new isolates and reference species representative of all trypanosomatid genera. ITS1 rDNA sequences, due to their

high polymorphism, were employed to assess inter- and intra-specific divergences. Jointly with light and electron morphological analyses, the molecular data supported the description of five new species of *Herpetomonas*, the inclusion in this genus of two new combinations and the exclusion of four organisms. Altogether, our findings prompted the revision of the genus *Herpetomonas*.

## Results

### Host Origin of *Herpetomonas* spp.

Of the 52 flagellates assigned to the genus *Herpetomonas* by V7V8 barcoding (see below), 90% and ~6.0% were recovered from dipterans and hemipterans respectively while ~4.0% were recovered from plants. The total dipteran sample studied included: Calliphoridae, 51%; Muscidae, 21%; Syrphidae, 11%; Sarcophagidae, 9.0%; Ceratopogonidae, 4.0%; Phoridae, 2.0% and Fanniidae, 2.0%. Insects of the families Tabanidae, Culicidae and Phlebotomidae tested negative for *Herpetomonas*, but these insects were not examined in sufficient numbers to provide confidence in the negative results. No specimens from the remaining dipteran families were examined (Table 1).

### Barcoding of Insect Trypanosomatids Using V7V8 SSU rRNA Sequences

We are currently barcoding the entire Trypanosomatid Culture Collection of the University of São Paulo (TCC-USP) by sequencing the V7V8 region of SSU rRNA. At present, the TCC-USP harbours more than 2,400 trypanosomatids, of which 527 are isolates from non-hematophagous insects, dipterans (244) and hemipterans (283). In this study, barcoding and the analysis of genetic relatedness using V7V8 rRNA permitted us to select 52 flagellates clustering with *Herpetomonas* spp. (Table 1), including two species formerly described as *Leptomonas*, *L. costoris* and *L. samueli*. Every culture has been cloned to ensure that characterized cultures corresponded to only one organism. In addition, the single PCR-amplified V7V8 rRNA sequences obtained also served to guarantee that cultures were from only one organism.

The dendrogram resulting from the analysis of the genetic relatedness among the reference species and the new isolates using V7V8 rRNA sequences revealed that the flagellates were distributed among 11 branches all comprising the presumed species of the genus *Herpetomonas* (Fig. 1). The V7V8 rRNA sequence divergences between isolates of a given species were small, averaging 0.6%, whereas sequence divergences between the type species *H. muscarum* and other *Herpetomonas* spp. averaged 14%. Isolates representative of all branches of *Herpetomonas* spp. disclosed by V7V8 rRNA sequences were subjected to phylogenetic analyses using gGAPDH and SSU rRNA sequences.

### Phylogenetic Inferences Based on SSU rRNA and gGAPDH Gene Sequences

Phylogenetic trees inferred using SSU rRNA and gGAPDH independent or concatenated sequences from 15 isolates from flies and three from hemipterans yielded similar topologies by Bayesian inference (BI) and maximum likelihood (ML) analyses (Fig. 2). In all trees, a strongly supported clade comprising new isolates and previously described species of *Herpetomonas* is clearly evident, indicating that this genus can be redefined as a monophyletic assemblage of flagellates, which now includes organisms previously classified in the genus *Leptomonas*, *L. samueli* and *L. costoris*, reclassified as *Herpetomonas* in this study.

Phylogenetic analyses of the gGAPDH and SSU rRNA genes confirmed the overall branching pattern disclosed by the V7V8 rRNA analysis. Together, the topology of phylogenetic trees and the genetic distances inferred using gGAPDH and SSU rRNA sequences allowed us to distinguish 13 species of *Herpetomonas.* Among these species, five are new species, such as the isolates TCC 266E, 1745, 1733, 1444 and 1982, which are being named *H. isaaci* n. sp., *H. puellarum* n. sp., *H. elegans* n. sp., *H. modestus* n. sp. and *H. wanderleyi* n. sp., respectively. Other flagellates correspond to known species, such as *H. muscarum/H. megaseliae, H. samuelpessoai, H. ztiplika*, *H. trimorpha* and *H. mariadeanei*. Finally, two branches represent former *Leptomonas* spp. reclassified as *Herpetomonas* in this paper: *H. samueli* n. comb. and *H. costoris* n. comb. The following species paired together: *H. samuelpessoai* and *H. samueli*, *H. puellarum* and *H. elegans, H. muscarum* and *H. isaaci, H. costoris* and *H. modestus, H. ztiplika* and *H. trimorpha*, and *H. wanderleyi* and *H. mirabilis*. *H. mariadeanei* was positioned separately in all phylogenetic trees (Fig. 2).

In all inferred trees, *H. mariadeanei* remains a solitary species apart from all the validated *Herpetomonas*. In the analyses based on the concatenated data set, this species was supported as the basal species of the *Herpetomonas* clade.

**Table 1.** Host and geographical origin of species of *Herpetomonas* and Genbank accession numbers of SSU rRNA, gGAPDH and ITS rDNA sequences.

| Trypano-somatid | TCC | Host origin | | | Geographical origin | date of isola-tion | Genbank acession number | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Order | Family | Species | City/State/country | | SSU rRNA | ITS1 rDNA | gGAPDH |
| *H. muscarum* | | | | | | | | | |
| | 001E | D | Mu | *Musca domestica* | Minneapolis/MN/USA | 1971 | JQ359731 | JQ406959/60/61 | JQ359748 |
| | 002E[a] | D | Ph | *Megaselia scalaris* | Lincoln/NE/USA | 1972 | JQ359715 | JQ406962/63/64 | JQ359749 |
| | 048E[b] | | | plant | Ocochobee/FL/USA | 1976 | JQ359686 | - | - |
| | 251E | D | Mu | *Musca domestica* | Itapetininga/SP/BR | 1988 | JQ359682 | - | - |
| | 257E | D | Mu | *Musca domestica* | Itapetininga/SP/BR | 1988 | JQ359683 | - | - |
| | 261E | D | Mu | *Musca domestica* | Arapongas/PR/BR | 1988 | JQ359684 | - | - |
| | 284E | D | Sy | *Ornidia obesa* | Itapetininga/SP/BR | 1988 | JQ359685 | - | - |
| *H. samuelpessoai* | | | | | | | | | |
| | 005E | H | Re | *Zelus sp.* | Goiânia/GO/BR | 1976 | JQ359716 | JQ406989/90/91 | JQ359743 |
| | 264E | D | Fa | *Fannia canicularis* | Rolândia/PR/BR | 1988 | JQ359688 | - | - |
| | 281E | D | Sa | *Sarcophaga sp.* | São Paulo/SP/BR | 1989 | JQ359689 | - | - |
| | 282E | D | Sa | *Sarcophaga sp.* | São Paulo/SP/BR | 1989 | JQ359717 | - | JQ359747 |
| | 325E[c] | | | plant | Alfenas/MG/BR | 2000 | JQ359687 | - | - |
| | 1443 | D | Ca | *Chrysomya megacephala* | Campo Grande/MS/BR | 2007 | JQ359692 | - | - |
| | 1545 | D | Mu | *Musca domestica* | Rolândia/PR/BR | 1988 | JQ359697 | - | - |
| | 1716 | D | Ca | *Lucilia sp.* | Bastos/SP/BR | 1989 | JQ359693 | - | - |
| | 1761 | D | Ca | *Chrysomya putoria* | Cufada/Buba/GB | 2010 | JQ359718 | JQ406992/93/94 | JQ359732 |
| | 1851 | D | Ca | *Chrysomya putoria* | Cufada/Buba/GB | 2010 | JQ359694 | - | - |
| | 1861 | D | Sa | *Peckia ingens* | São Paulo/SP/BR | 2008 | JQ359695 | - | - |
| | 1870 | D | Sa | *Peckia ingens* | São Paulo/SP/BR | 2008 | JQ359696 | - | - |
| *H. isaaci* | | | | | | | | | |
| | 266E | D | Mu | *Musca domestica* | Rolândia/PR/BR | 1988 | JQ359720 | JQ406965/66/67 | JQ359745 |
| | 270 | D | Ca | *Chrysomya albiceps* | Alfenas/MS/BR | 1999 | JQ359709 | - | - |
| | 276E | D | Ca | *Chrysomya putoria* | Bastos/SP/BR | 1988 | JQ359721 | JQ406968/69/70 | JQ359746 |
| | 278E | D | Ca | *Chrysomya megacephala* | São Paulo/SP/BR | 1989 | JQ359690 | - | - |
| | 1533 | D | Mu | *Musca domestica* | Rolândia/PR/BR | 1988 | JQ359702 | - | - |
| | 1699 | D | Ca | *Chrysomya putoria* | Bastos/SP/BR | 1988 | JQ359703 | - | - |
| | 1724 | D | Sy | *Ornidia obesa* | Monte Negro/RO/BR | 2008 | JQ359704 | - | - |
| | 1766 | D | Ca | *Chrysomya putoria* | Cufada/Buba/GB | 2010 | JQ359698 | - | - |
| | 1767 | D | Ca | *Chrysomya megacephala* | Monte Negro/RO/BR | 2008 | JQ359706 | - | - |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1881 | D | Ca | *Chrysomya megacephala* | Confresa/MT/BR | 2009 | JQ359699 | - | - |
| | 1882 | D | Ca | *Chrysomya megacephala* | Canfresa/MT/BR | 2009 | JQ359700 | - | - |
| | 1885 | D | Ca | *Chrysomya megacephala* | Confresa/MT/BR | 2009 | JQ359707 | - | - |
| | 1852 | D | Ca | *Chrysomya albiceps* | São Paulo/SP/BR | 2009 | JQ359710 | - | - |
| | 1941 | D | Ca | *Lucilia eximia* | Miranda/MS/BR | 2009 | JQ359708 | - | - |
| | 1944 | D | Ca | *Chrysomya megacephala* | Miranda/MS/BR | 2009 | JQ359701 | - | - |
| | 2026 | D | Ca | *Chrysomya putoria* | Cufada/Buba/GB | 2010 | JQ359719 | - | JQ359733 |
| | 2069 | D | Ca | *Chrysomya megacephala* | Campo Grande/MS/BR | 2010 | JQ359705 | - | - |
| *H. puelarum* | | | | | | | | | |
| | 1745 | D | Ca | *Chrysomya putoria* | Cufada/Buba/GB | 2110 | JQ359723 | JQ406971/72/73 | JQ359734 |
| | 247E | D | Mu | *Musca domestica* | Cotia/SP/BR | 1988 | JQ359724 | JQ406974/75/76 | JQ359744 |
| | 254E | D | Mu | *Musca domestica* | Itapetininga/SP/BR | 1988 | JQ359691 | - | - |
| *H. elegans* | | | | | | | | | |
| | 1733 | D | Sy | *Ornidia obesa* | Monte Negro/RO/BR | 2008 | JQ359725 | JQ406977/78/79 | JQ359735 |
| | 1727 | D | Sy | *Ornidia obesa* | Monte Negro/RO/BR | 2008 | JQ359711 | - | - |
| | 1848 | D | Sy | *Ornidia obesa* | São Paulo/SP/BR | 2009 | JQ359712 | - | - |
| *H. modestus* | | | | | | | | | |
| | 1444 | D | Ca | *Chrysomya megacephala* | Campo Grande/MS/BR | 2007 | JQ359726 | JQ406980/81/82 | JQ359736 |
| | 1943 | D | Ca | *Cochliomyia macellaria* | Miranda/MS/BR | 2009 | JQ359727 | - | JQ359737 |
| | 2049 | D | Ca | *Chrysomya megacephala* | Campo Grande/MS/BR | 2010 | JQ359713 | | |
| *H. costoris* | 019E | D | Ge | *Gerris comatus* | Minnetonka/MN/USA | 1965 | JQ359728 | JQ406983/84/85 | JQ359738 |
| *H. samueli* | 003E | H | Re | *Zelus leucogrammus* | Goiânia/GO/BR | 1964 | JQ359722 | JQ406986/87/88 | JQ359742 |
| *H. mirabilis* | 301E | D | Ca | *Cynomyopsis cadaverina* | Chichicastenango/GT | 1964 | JQ359729 | JQ417275 | JQ359739 |
| *H. wanderleyi* | 1982 | D | Ca | *Cochliomyia macellaria* | Miranda/MS/BR | 2009 | JQ359730 | JQ417274 | JQ359740 |
| *H. mariadeanei* | 004E | D | Mu | *Muscina stabulans* | São Paulo/SP/BR | 1978 | JQ359714 | JQ406956/57/58 | JQ359741 |
| *H. trimorpha* | | D | Ce | *Culicoides truncorum* | Mikulov/Moravia/CZ | 2010 | EU179326** | | FJ850241** |

Table 1 (*Continued*)

| Trypanosomatid | TCC | Host origin | | | Geographical origin | date of isolation | Genbank acession number | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Order | Family | Species | City/State/country | | SSU rRNA | ITS1 rDNA | gGAPDH |
| *H. ztiplika* | | D | Ce | *Culicoides kibunensis* | Mikulov/Moravia/CZ | 2004 | AF416560** | | FJ850242** |

TCC (code number) - Trypanosomatid Culture Collection, University of São Paulo, SP, Brazil. H, Hemiptera; D, Diptera; P, Plant Insect families: Ca, Calliphoridae; Fa, Fanniidae; Ge, Gerridae; Mu, Muscidae; Ph, Phoridae; Re, Reduviidae; Sa, Sarcophagidae; Sy, Syrphidae.
[a]TCC002E: former *Herpetomonas megaseliae*;
[b]TCC048E: former *Phytomonas davidi*;
[c]TCC325E: former *H. samuelpessoai camargoi*.
BR, Brazil; GB, Guinea Bissau; GT, Guatemala, CZ, Czech Republic; MN, Minnesota; NE, Nebraska; FL, Florida; SP, São Paulo; PR, Paraná; GO, Goiás; MG, Minas Gerais; MS, Mato Grosso do Sul; RO, Rondônia; MT, Mato Grosso.
**Sequences not determined in this study.

However, in independent analyses using the SSU rRNA and gGAPDH genes, the positioning of *H. mariadeanei* varied, and it was sometimes placed outside of the strongly supported clade harbouring all other species of *Herpetomonas* (data not shown). Other problematic species are *H. ztiplika, H. trimorpha, H. mirabilis* and *H. wanderleyi* since their positioning in the phylogenetic trees was unresolved, fluctuating according to the genes, species composition and methodology employed for tree inferences. However, unlike *H. mariadeanei*, these species always remained within the *Herpetomonas* clade. The average divergences between *Herpetomonas* species found using the SSU rRNA and gGAPDH were large, corresponding to ~7.0% and 13%, respectively. The inter-specific divergences of gGAPDH sequences, which showed largest discriminating power, varied from 2.0% to 24%, whereas isolates belonging to any given species of *Herpetomonas* diverged by less than 1.0% (Supplementary Table S1).

## Polymorphism of the ITS1 rDNA Gene Sequences

Within the Trypanosomatidae, due to their high divergence compared to SSU rRNA and gGAPDH, ITS rDNA sequences have been used for diagnostic and phylogenetic inferences at species and lineage levels (Dollet et al. 2012; Lima et al. 2012; Teixeira et al. 2011). ITS rDNA sequences may be inappropriate for phylogenetic analyses of distantly related species due to excessive polymorphism and problematic nucleotide alignments. However, conserved structural elements within the secondary structures of ITS permit to refine the alignment, thus providing a well-supported background for the phylogenetic application of ITS primary and secondary structures to address phylogenetic and taxonomic questions (Letsch et al. 2010; Miao et al. 2008; Ponce-Gordo et al. 2011; Schultz and Wolf 2009; Sun et al. 2010; Thornhill and Lord 2010).

Here, we analysed both primary and putative secondary structures of 11 species of *Herpetomonas* demonstrating the value of the ITS rDNA for intra-generic polymorphism analysis. Sequences from ITS1 rDNA of *Herpetomonas* spp. largely varied in length, from 145 to 770 bp (Fig. 3), with *H. mirabilis* and *H. wanderleyi* showing exceptionally long ITS1 rDNA, 615 and 770 bp respectively, which were excluded from the final alignment containing all other species due to extensive sequence differences, including large gaps, which precluded a straightforward alignment. The branching pattern of the dendrogram constructed

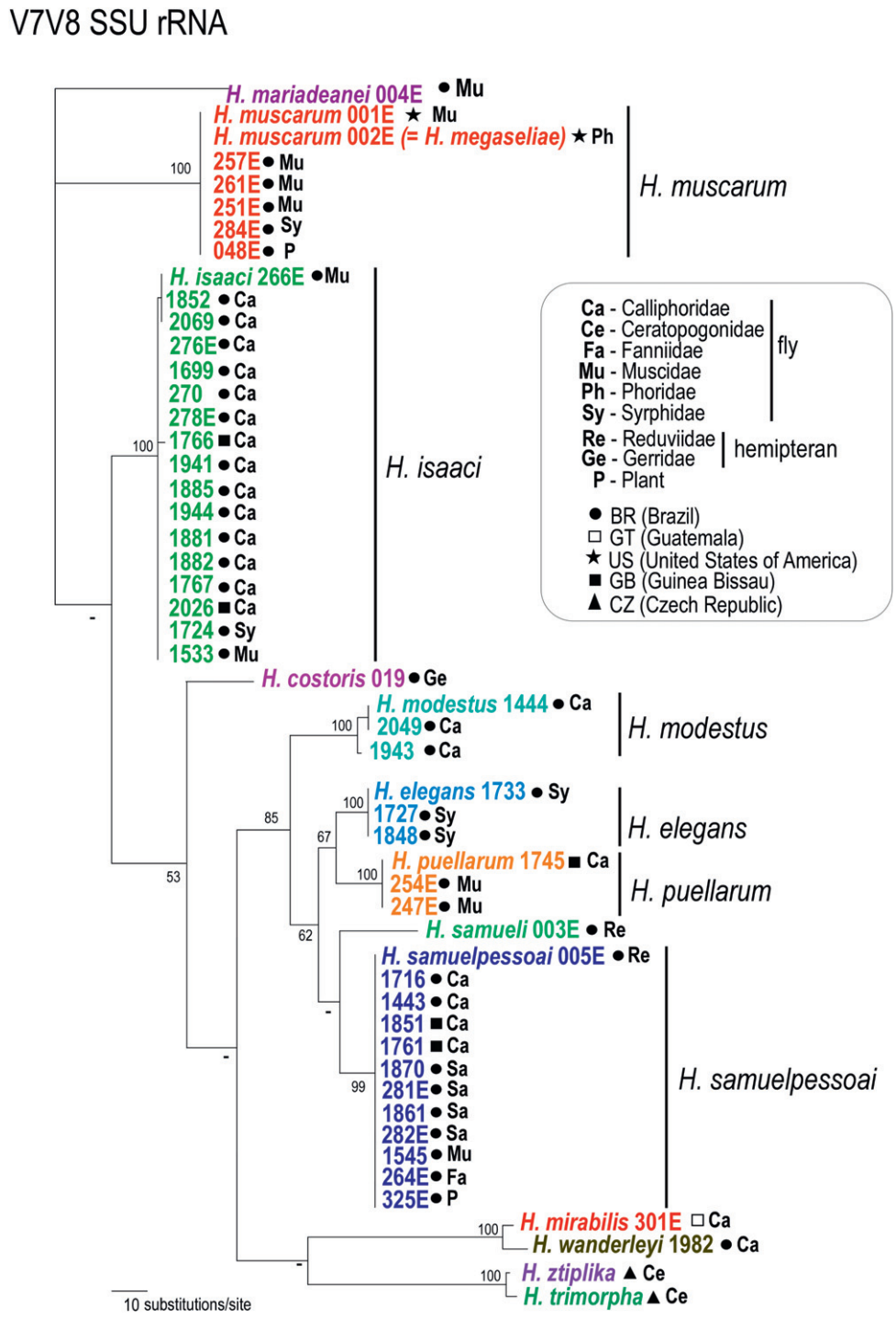



**Figure 1.** Dendrogram inferred by parsimony analysis using 52 sequences from V7V8 SSU rRNA (alignment A1, 766 characters, CI=0.7; RI=0.9; number of steps of the single tree obtained = 432) from species of *Herpetomonas* and new isolates of insect trypanosomatids that share relevant similarity with sequences from reference-species of *Herpetomonas* (BLAST search). The numbers at nodes correspond to percentage of bootstrap values derived from 100 replicates (- support value <50%). The accession numbers of sequences in Genbank are in Table 1.

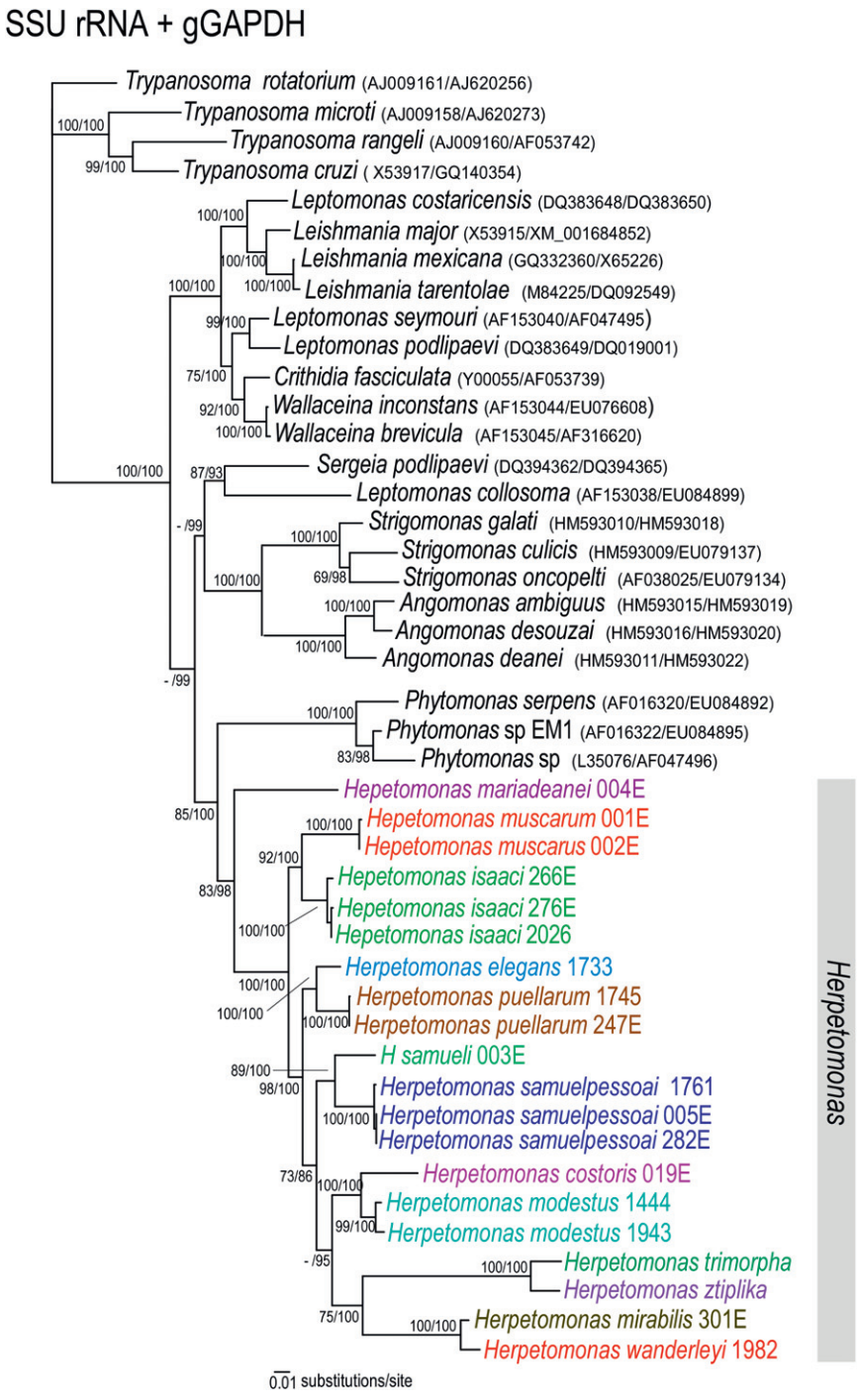



**Figure 2.** Phylogenetic tree of *Herpetomonas* spp. and representatives of distinct trypanosomatid genera inferred by maximum likelihood (ML) analysis based on concatenated small subunit (SSU) rRNA and glycosomal glyceraldehyde-3-phosphate dehydrogenase (gGAPDH) gene sequences (alignment A4, 2796 characters, Ln = -20011.290293). The accession numbers of SSU rRNA and gGAPDH sequences of *Herpetomonas* spp in Genbank are in Table 1, and those of the remaining organisms are in the tree within parenthesis. Numbers at the major nodes correspond respectively to ML and BI support values (- support value < 50%).

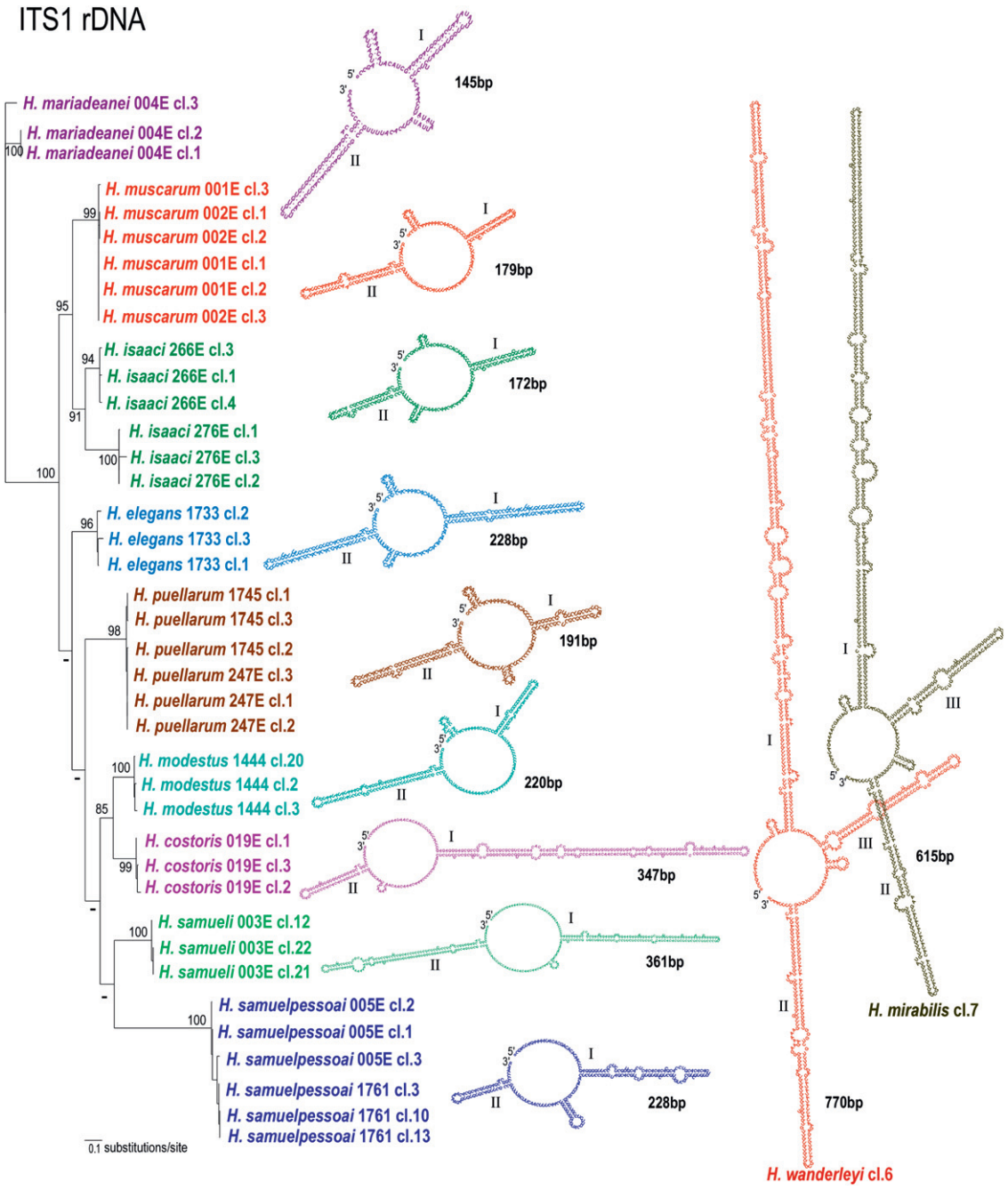



**Figure 3.** Dendrogram inferred by maximum likelihood (ML) of the ITS1 rDNA sequences (646 characters, Ln= -2642.432156) from 9 species of *Herpetomonas* (accession numbers in Genbank are in Table 1). Values indicated at the nodes correspond to bootstrap support derived from 500 replicates (- support value < 50%). Length of ITS1 rDNA sequences varied from 145 to 770 bp. Putative secondary structures (thermodynamically predicted at 37°C using the UNAFold software) of ITS1 region from 11 *Herpetomonas* spp. showing the open loop with two-three main structural domains (helices I-III) and small accessory helices. The estimated values of thermodynamic energy of modelled structures (ΔG Kcal/mol): *H. mariadeanei* (-54.6), *H. muscarum* (-45.7), *H. isaaci* (-42.5), *H. elegans* (-70.9), *H. puellarum* (-63.6), *H. costoris* (-129.1), *H. modestus* (-68.5), *H. samueli* (-138.4), *H. samuelpessoai* (-63.9), *H. mirabilis* (-281.3) and *H. wanderleyi* (-335.5).

using ITS1 rDNA sequences of *Herpetomonas* spp. (Fig. 3) concurs with the topologies of SSU rRNA and gGAPDH phylogenetic trees. The ITS1 rDNA sequence divergences between the species of *Herpetomonas* that could be included in the alignment ranged from ~ 40 to 77%. Divergences between sequences of isolates of a given species were always much smaller than those between sequences from distinct species, ranging from ~1.0% to 11%. Therefore, ITS1 rDNA sequences proved valuable to discriminate species within *Herpetomonas*.

In agreement with large sequence and length variability of ITS1, the species of *Herpetomonas* showed quite variable predicted secondary structure models. It has been reported that ITS1 secondary structure show a conserved core structure with an open loop and different numbers of helices (Ponce-Gordo et al. 2011; Sun et al. 2010; Thornhill and Lord 2010). Putative secondary structure models of ITS1 from *Herpetomonas* disclosed the general main features: a large open loop with two main helices (I and II) sharing conserved regions and one or two small accessory helices in most species. Exceptions were *H. wanderleyi* and *H. mirabilis* that showed an additional large helix (III); these two species displayed the longest helices bearing several bulge loops. Helices varied in number and length among the different species thus yielding species-specific secondary structures (Fig. 3). The predicted secondary structures of ITS1 allowed differentiation of all the 11 species investigated, including *H. wanderleyi* and *H. mirabilis* that could not be included in the primary structure alignments (Fig. 3). Analysis of two isolates of each species yielded identical overall structure models, thus confirming their value for *Herpetomonas* species identification.

## Morphological Characterization by Light and Scanning Electron Microscopy (SEM)

Examination of Giemsa-stained preparations of log-phase cultures of *Herpetomonas* by light microscopy revealed inter- and intra-specific differences in the length and shape of the flagellates. Polymorphism is inherent to *Herpetomonas* spp., but it is further complicated by morphological variability influenced by the culture medium and phase of growth. Thus, attempts to distinguish species by morphological characteristics are ineffectual. Intraspecific polymorphism was detected in cultures that were cloned and re-cloned. Therefore, marked polymorphism of *Herpetomonas* spp. cannot be attributed to a mixture of different species, a fact that was confirmed by sequencing of SSU rRNA and gGAPDH genes, which yielded single sequences for each culture.

The variation in body size amongst promastigotes of the various species of *Herpetomonas* is represented in Figure 4. The same polymorphism described by light microscopy was observed by SEM (Figs 5, 6). Promastigotes of *H. puellarum* (Fig. 5a, e) and *H. elegans* (Fig. 5d, h) are most regularly oblong and constitute the smallest flagellates of *Herpetomonas* (Fig. 4). Many promastigotes of these species exhibit a vase-like and funnelled anterior end resembling choanomastigotes, while others have a sharply truncated posterior end. Few opisthomastigotes are observed in *H. puellarum*, whereas *H. elegans* present many opisthomastigotes. Promastigotes of *H. samuelpessoai* (Fig. 5b, f) are larger than those of the above species and generally have rounded posterior ends. *H. isaaci* has wider promastigotes than those of *H. samuelpessoai* and lower numbers of opisthomastigotes (Fig. 5c, g). Promastigotes of *H. modestus* (Fig. 5i, j) are quite heterogeneous in size and, in general, display a pointed posterior end; opisthomastigotes of this species are short and bulgy (Fig. 5i).

In morphological terms, the archetype of the genus *Herpetomonas* is *H. muscarum*, which promastigotes are generally homogeneous, long and needle-like, with sharp posterior ends and long flagella (Fig. 5k, o). Opisthomastigotes of this species are slim and large (Fig. 5k). Pro- and opisthomastigotes of this species correspond to the classical *Herpetomonas* forms described by Hoare and Wallace (1966). In *H. muscarum*, opisthomastigotes are always present at percentages larger than in any other species. Promastigotes of *H. muscarum* (Fig. 5k, o) and *H. samueli* (Fig. 5l, p) have a certain degree of morphological resemblance. *H. costoris* (Fig. 5m, n) is polymorphic and presents a majority of medium sized promastigotes alongside twisted flagellates with a wide body and long, tapered posterior ends. Promastigotes of *H. mariadeanei* (Fig. 5q) resemble those of *H. muscarum*, but their opisthomastigotes are very peculiar, showing a long intracellular segment of the flagellum (Fig. 5r), which gave them the name of endomastigotes. Detailed morphological data about this species are in Yoshida et al. (1978).

*H. mirabilis* and *H. wanderleyi* are extremely polymorphic flagellates varying in shape, body length and width, and length of free flagellum (Figs 4, 6). Extremely large promastigotes can be observed alongside medium and short promastigotes. Some promastigotes are very long and filiform (Fig. 6a), with the kinetoplast located far from the nucleus

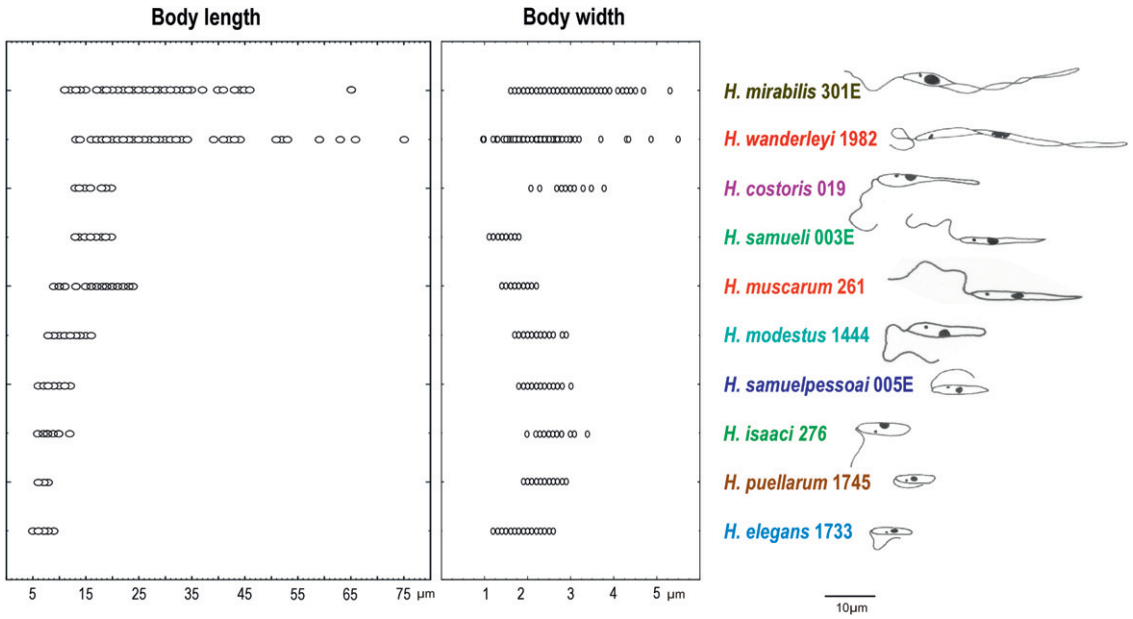


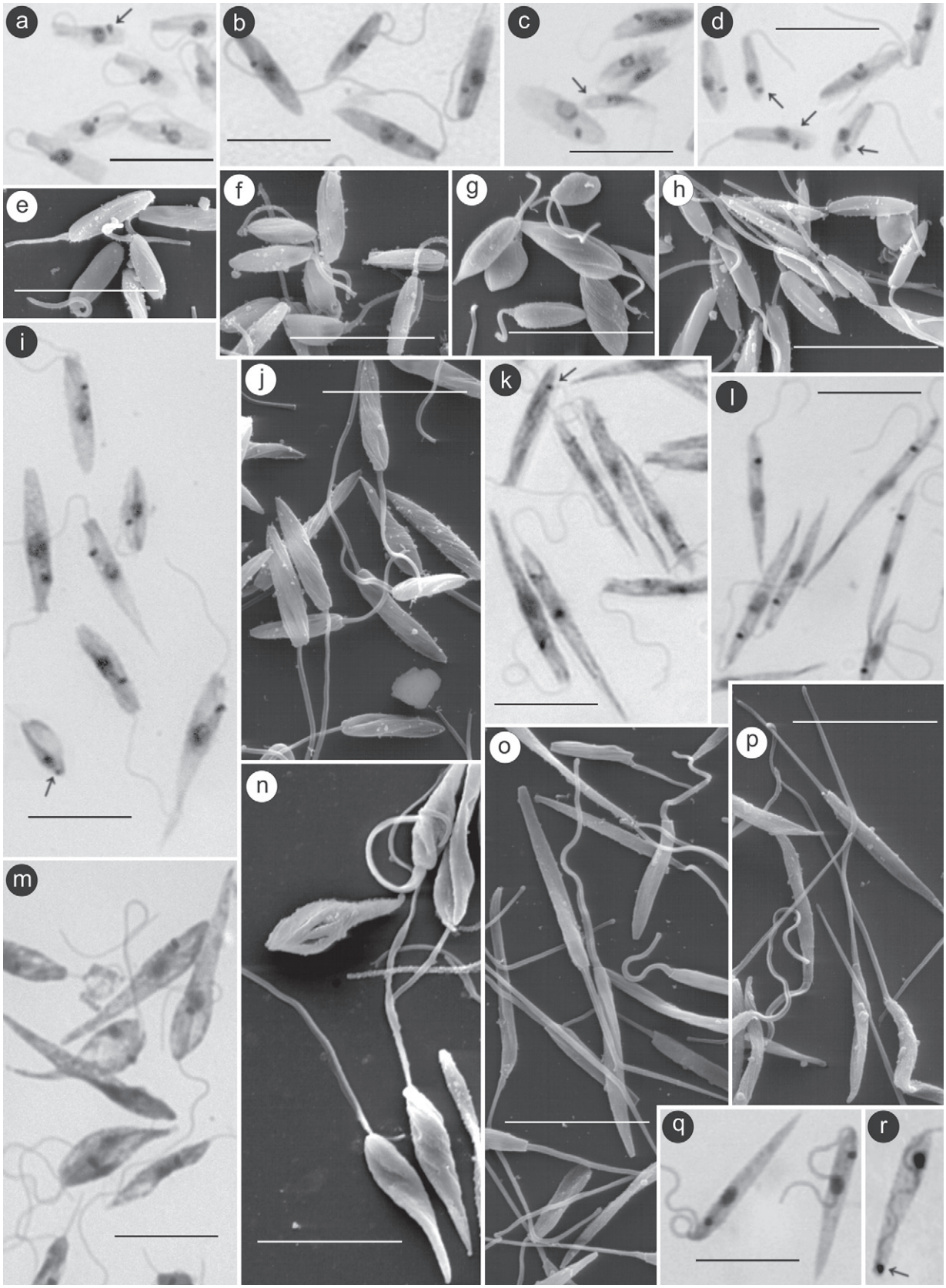

**Figure 4.** Morphological variability of promastigotes of *Herpetomonas* species demonstrated by the large dispersion of both body length and width of ~30 flagellates of each species. The graphic scatter plot was made with the Statsoft Program. Side drawings illustrate flagellates representative of each species, Bars=10 µm.

(Fig. 6d) while others have twisted bodies (Fig. 6c, d, e). Large promastigotes of *H. mirabilis* present a shape reminiscent of tadpoles, with large heads and long, thin and many times twisted tails (Fig. 6f - i). The measurements shown in Figure 4 reflect the extreme polymorphism of *H. mirabilis* and *H. wanderleyi.* The promastigotes of *H. mirabilis* and *H. wanderleyi* are larger than those reported for *H. ztiplika* and *H. trimorpha* (Podlipaev et al. 2004a; Zídková et al. 2010) and for *Phytomonas* spp. (Camargo 1998).

The pleomorphism within species and isolates of the same species and the lack of sharply definable species characteristics thwart the diagnostic value of morphology for the taxonomy of *Herpetomonas*. In addition, the absence of opisthomastigotes in many species invalidates the long-held tenet that opisthomastigotes are the hallmark of this genus.

## Ultrastructural Analysis by Transmission Electron Microscopy (TEM)

Log-phase cultures of seven distinct species of *Herpetomonas*, including new and reference-species, were morphologically compared by TEM. The sample included species characterized by small (Fig. 7i), intermediary (Fig. 7e) and very large promastigotes (Fig. 7a). The common organelles and structures of the Trypanosomatidae were observed in all the *Herpetomonas* species examined. Two structures deserve detailed comments: the spongiomes and the paraflagellar rod (PFR). Spongiomes are constituted by numerous tubules (Fig. 7c, f, g) that converged on the contractile vacuoles attached to the membrane of the flagellar pocket (Fig. 7f, g) constituting the osmoregulatory apparatus (Rohloff and Docampo 2008). The PFR is a complex trilaminar network of filaments unique of kinetoplastid flagellum that runs alongside the axonemal microtubules, whose arrangement varied in different trypanosomatids (De Souza and Souto-Padron 1980; Portman and Gull 2010). The PFR showed to be well-developed in *H. megaseliae* (De Souza and Souto-Padron 1980) and *L. costaricensis* (Yurchenko et al. 2006b), and extremely developed in *L. barvae* (Maslov et al. 2010). In all *Herpetomonas* spp. examined, PFRs are easily visible, inclusively at the portion of the flagellum located inside the flagellar pocket (Fig. 7b, f, k). Filaments of PFR are longitudinally oriented in relation to the axoneme and crossed by oblique filaments forming a lattice-like network (Fig. 7j). The species of *Herpetomonas* exhibit a large flagellar pocket (Fig. 7b, c, e - g, i) with hemidesmosomes linking a swelling of the flagellum to the plasma membrane of the flagelllar pocket (Fig. 7b, c), and well-developed mitochondria (Fig. 7a, b, d) with exuberant cristae (Fig. 7d). Acidocalcisomes are observed in small numbers as vacuoles empty or with electron-dense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inclusions (Fig. 7b - f, h). Glycosomes, which appear as electron-dense, round or oval organelles (Fig. 7a, b, d), and poorly developed Golgi complexes (Fig. 7 g) are also present in all species examined. No endosymbionts were detected in the *Herpetomonas* spp. examined.

The kinetoplasts (formed by concatenated molecules of mini- and maxicircles of mitochondrial DNA) display rather uniform morphological structure in the *Herpetomonas* spp. examined. They appear in longitudinal sections as condensed rod-shaped structures (Fig. 7b, e, g - i, l), whose thickness varies from 120 to 230 nm, averaging 156 nm. In *H. samuelpessoai*, kDNA disk thickness varied ~60 nm at different culture phases. We could not ascertain whether this variability was attributable to cell cycle changes or to the differentiation process of promastigotes into opisthomastigotes. The kDNA thickness can vary among species of trypanosomatids according to the size of kDNA minicircles (Lukeš and Votýpka 2000; Teixeira et al. 2011). However, in *Herpetomonas*, the kinetoplast thickness does not exhibit relevant inter-specific variability.

This is the first time that old and newly assigned species of *Herpetomonas*, cultivated under the same conditions, are compared by light microscopy, SEM and TEM. TEM revealed an overall similar ultrastructural organization for all the species examined, even though they strongly diverged when examined by light microscopy and SEM. For the present reappraisal of *Herpetomonas* it was essential to report on the general ultrastructural organization of its species, as has been usually done in the description of genera and species of trypanosomatids in general (Lima et al. 2012; Maslov et al. 2010; Merzlyak et al. 2001; Teixeira et al. 2011; Viola et al. 2009; Yurchenko et al. 2006a, b; Zídková et al. 2010).

## Taxonomy Section: Amendments and New *Herpetomonas* Species

### Genus *Herpetomonas* Kent, 1880

**Redefinition and Diagnosis**: The genus *Herpetomonas* is herein redefined as a monophyletic group of flagellates defined by phylogenetic analyses based on gGADPH and SSU rRNA sequences from a representative sample of flagellates of all trypanosomatid genera. In inferred phylogenetic trees based on these gene sequences, *Herpetomonas* emerges as a clearly defined clade. Morphologically, this genus comprises extremely polymorphic promastigotes varying from very small to very large flagellates. In addition to promastigotes, species of this genus may or may not present a small percentage of opisthomastigotes, opisthomorphs or endomastigotes. Dipterans are the preferential hosts of *Herpetomonas* spp. *Herpetomonas muscarum* remains the type species of the redefined genus. **Comments**: Due to the absence of opisthomastigotes in several phylogenetically validated species of *Herpetomonas*, the presence of pro- and opisthomastigotes in cultures can no longer be the defining criterion for the genus *Herpetomonas* as proposed by Hoare and Wallace (1966).

#### 1. Currently Valid, Cultivated Species of *Herpetomonas*

In the literature, there are numerous species of *Herpetomonas*, however, most species are not available in culture and, thus, are not amenable to molecular analysis. In this taxonomic section, we consider exclusively the species of *Herpetomonas* included in molecular phylogenetic analyses and available in culture collections.

#### 1.1. ***Herpetomonas muscarum*** (Leid, 1856; Kent, 1880) Rogers and Wallace, 1971

Since the 19th century, flagellates from the housefly have been described and re-described as *H. muscarum* by various authors in diverse geographic regions (Wallace, 1966). The currently available culture was isolated by Rogers and Wallace (1971) in Minnesota, USA. **Type material:** hapantotype, culture ATCC 30260, of which culture TCC001E deposited at Trypanosomatid Culture Collection of the University of São Paulo (TCC-USP) is a duplicate. Paratypes: cultures of isolates TCC048E, 251E, 257E, 261E, 284E deposited at TCC-USP and ATCC 30287. **Type host**: *Musca domestica*, Diptera, Muscidae. The flagellates from these cultures are similar to *H. muscarum* in all phylogenetic analyses but may have been collected at different localities and from different hosts, as shown in Table 1. **Morphology:** This species displays the classical morphology of the genus *Herpetomonas*. Promastigotes are elongated and needle-like (Fig. 5 k, o). Giemsa-stained glass smears are deposited at TCC-USP. Measurements of cultured promastigotes: body length, 9.3 – 24.4 μm (16 ± 4.9); body width, 1.4 – 2.0 μm (1.8 ± 0.2 μm); free flagellum 3.0 – 26 μm (16.5 ± 8 μm). Opisthomastigotes occur in variable numbers (up to 15%) depending on culture conditions. **Diagnosis**: Based on sequences deposited in Genbank from SSU rRNA (JQ359731), gGAPDH (JQ359748) and ITS1 rDNA (JQ406959-61). **Comments**: *Phytomonas davidi* McGhee and Postell (1976) was isolated from the spurge *Euphorbia heterophylla* and named after the homonymous *Phytomonas davidi* (Donovan 1909). The culture was deposited as ATCC30287. We received a duplicate (TCC048E) from McGhee, which was morphologically indistinguishable from *H. muscarum* and their

**Figure 5.** Light and scanning microscopy of new and reference species of *Herpetomonas*. Optical microscopy (**a-d,i, k-m, q, r**) and scanning electron microscopy (SEM) (**e-h, j, n-p**) of flagellates from logarithmic cultures showing promastigotes of *H. puellarum* (**a, e**), *H. samuelpessoai* (**b, f**), *H. isaaci* (**c, g**), *H. elegans* (**d, h**), *H. modestus* (**i, j**), *H. muscarum* (**k, o**), *H. samueli* (**l, p**), *H. costoris* (**m, n**) and *H. mariadeanei* (**q, r**). Arrows point to opisthomastigotes. Bars = 10 μm.

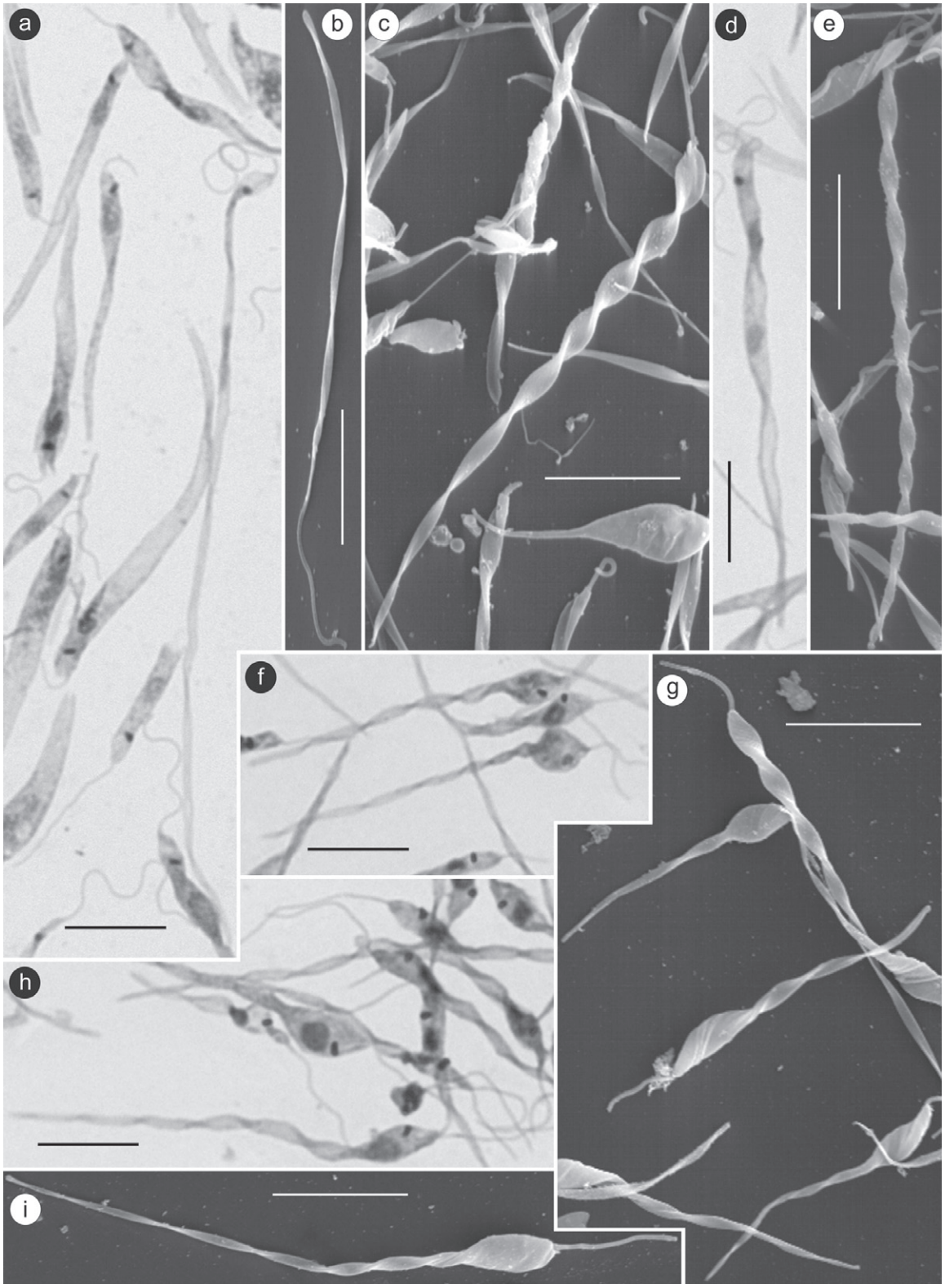



**Figure 6.** Light (**a, d, f, h**) and scanning electron microscopy (**b, c, e, g, i**) of promastigotes from logarithmic cultures of *H. wanderleyi* (**a-e**) and *H. mirabilis* (**f-i**). Bars = 10 μm.

SSU rRNA sequences were nearly identical to those of *H. muscarum*. Accordingly, we designate TCC048E and ATCC30287 as paratypes of *H. muscarum.*

**Synonymy** (heterotypic): *H. megaseliae* Dagget et al. 1972. This species was isolated by Dagget et al. (1972) in Lincoln, Nebraska, USA, from *Megaselia scalaris*, Muscidae, Diptera. This species is morphologically indistinguishable from *H. muscarum* and SSU rRNA and gGAPDH sequences of these two species diverge only 0.1% and 0.3% respectively. We propose that this species is a subjective junior synonym of *H. muscarum.*

### 1.2. ***Herpetomonas samuelpessoai*** (Carvalho 1973)

Roitman et al. 1976. Diverse trypanosomatids were present in a mixed culture that originated from a predator hemipteran in Goiás, Brazil (Carvalho 1973). Among these flagellates, Roitman et al. (1976) isolated a pure culture of *H. samuelpessoai*. **Type material:** hapantotype, culture deposited as ATCC30252 of which TCC005E deposited at TCC-USP is a duplicate. Paratypes: cultures TCC264E, 281E, 282E, 325E, 1716, 1443, 1545, 1761, 1851, 1861 and 1870 deposited at TCC-USP. These isolates are highly similar to *H. samuelpessoai* in all compared DNA sequences despite having different hosts collected at different localities (Table 1). **Type host:** *Zelus leucogrammus*, Hemiptera, Reduviidae. However, the actual host could be a fly on which the predator fed. **Morphology:** Promastigotes are smaller, wider and shorter than those of *H. muscarum*. Opisthomastigotes are present in cultures at 1-5%, depending on the medium composition and growth phase (Fig. 5b, f). Giemsa-stained glass smears are deposited at TCC-USP. Measurements of promastigotes: body length, 6.3 – 11.6 μm (9.0 ± 1.5 μm); body width 1.8 – 3.0 μm (2.3 ± 0.3 μm); free flagellum 6.6 – 14.3 μm (10.7 ± 2.2 μm). **Diagnosis**: DNA sequences deposited in Genbank from SSU rRNA (JQ359716), gGAPDH (JQ359743) and ITS1 rDNA (JQ406989-91).

**Comments**: Recent papers referred to *H. samuelpessoai* as *Herpetomonas pessoai* after Merzlyak et al. (2001) adopted a proposition from Podlipaev (1990). According to historical records, this was a mistaken proposition. *H. pessoai* was described from dipterans (*Anopheles* spp.) by Galvão and Coutinho (1941), whereas *H. samuelpessoai* was described 35 years later from the hemipteran *Zelus* sp. by Roitman et al. (1976). The morphology of these flagellates is completely distinct in their respective original descriptions. *H. pessoai* was never cultivated. Both species were named to honour Prof. Samuel B. Pessôa; Roitman et al. (1976) adopted *H. samuelpessoai* because *H. pessoai* was preoccupied. Therefore, they are distinct species and the name *H. samuelpessoai* should prevail for the species deposited as ATCC30252 and TCC005E.

**Synonymy** (heterotypic): *Herpetomonas samuelpessoai camargoi* Fiorini et al. (2001) is being designated a subjective junior synonym of *H. samuelpessoai* because SSU rRNA and gGAPDH sequence divergences between these flagellates are negligible (<0.1%). *H. samuelpessoai camargoi* was designated a subspecies mainly because the presence of a bifid posterior end in some promastigotes. However, this trait can be observed in other species of *Herpetomonas* and in trypanosomatids of other genera. In addition, the differences in RAPD (randomly amplified polymorphic DNA) profiles used to distinguish *H. samuelpessoai camargoi* (Fiorini et al. 2001) are compatible with a different genotype of *H. samuelpessoai.*

### 1.3. ***Herpetomonas mirabilis*** (Roubaud 1908; Wallace and Todd 1964) Wenyon, 1926

This flagellate was isolated by Wallace and Todd (1964) from a blowfly in Guatemala. **Type material:** hapantotype, culture deposited as ATCC 30263 of which TCC301E deposited at TCC-USP is a duplicate. Giemsa-stained glass smears are deposited at TCC-USP. **Type host**: *Cynomyopsis cadaverina*, Diptera Calliphoridae. **Locality**: Chichicastenango, Guatemala. **Morphology:** Polymorphic promastigotes, including very long promastigotes with twisted bodies. No opisthomastigotes were reported (Fig. 6f - i). Measurements of promastigote: body length, 11.4 – 65.2 μm (29 ± 9.1 μm); body width 1.6 – 4.4 μm (2.8 ± 0.8 μm) and free flagellum 2.7– 21.5 μm (10.3 ± 4.5 μm). **Diagnosis:** Based on DNA sequences deposited in Genbank: SSU rRNA (JQ359729), gGAPDH (JQ359739) and ITS1 rDNA (JQ417275). **Comments:** Roubaud first described similar extremely large flagellates in a blowfly in the Congo in 1908. Wenyon (1926) placed the similar flagellates reported by different authors in blowflies from India, Italy and Sudan in the genus *Herpetomonas.* Wallace and Todd (1964) named similar extremely large flagellates in blowflies of Guatemala as *Leptomonas mirabilis*. The present study demonstrated that this flagellate is in fact a *Herpetomonas* species, giving priority to the designation of *H. mirabilis* proposed by Wenyon (1926).

### 1.4. ***Herpetomonas ztiplika*** Podlipaev et al. 2004

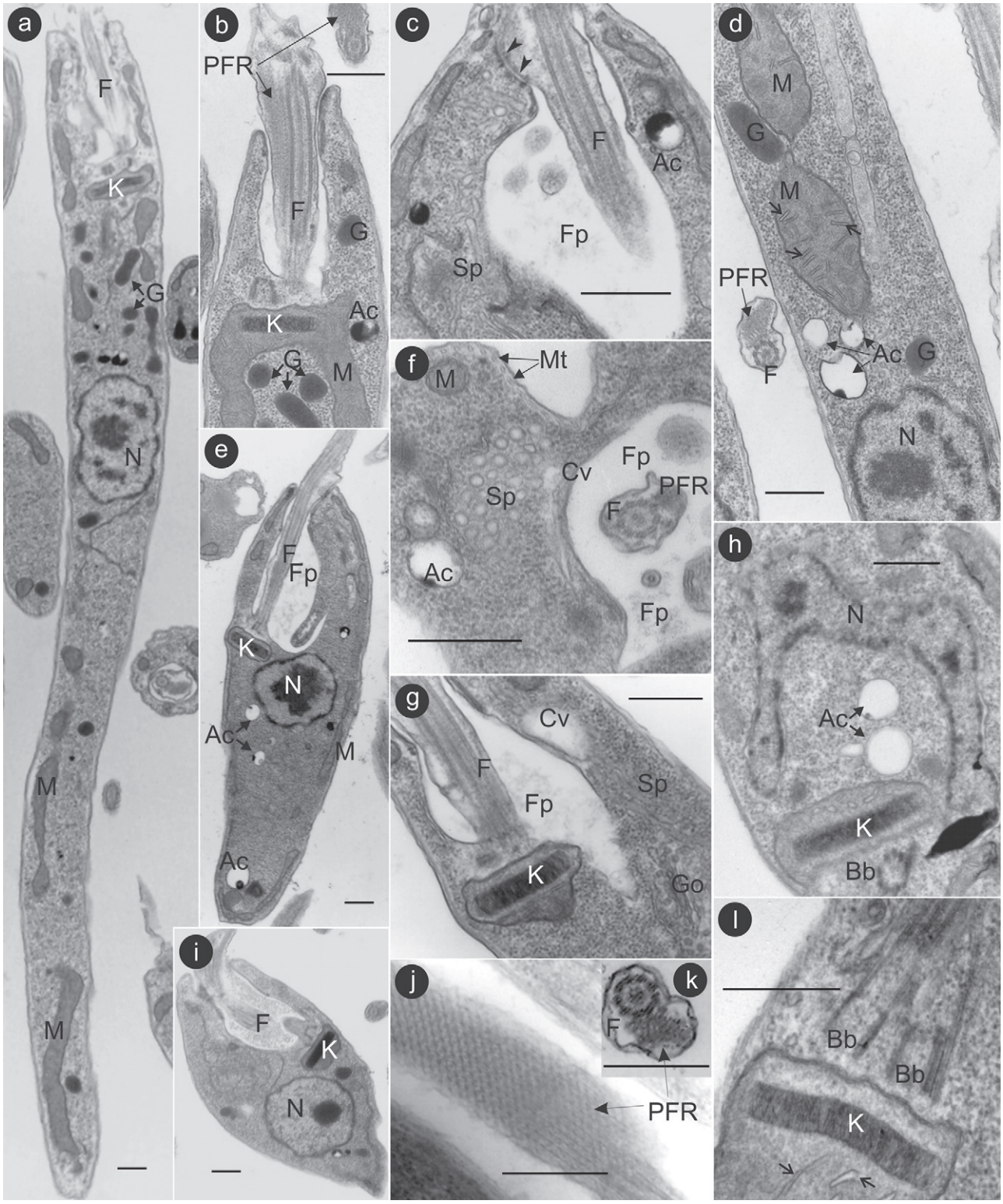
This flagellate was isolated by Podlipaev et al. (2004a) from a biting midge. **Type material:** hapantotype, culture 1CUL/CZ/1999/CER1 deposited at the Culture Collection of Charles University, Prague, Czech Republic. There are no paratypes designated for the species. **Type host**: *Culicoides kibunensis*, Diptera, Ceratopogonidae. **Locality**: Moravia, Czech Republic. **Morphology**: Large and polymorphic promastigotes Measurements in Podlipaev et al. (2004a). No opisthomastigotes have been reported. **Diagnosis:** SSU rRNA and gGAPDH gene sequences (Table 1) deposited in Genbank by Podlipaev et al. (2004a).

### 1.5. ***Herpetomonas trimorpha*** Zídková et al. 2010

This flagellate was isolated by Zídková et al. (2010) from a biting midge. **Type material:** hapantotype, culture ICUL/CZ/2001/CER9 deposited at the Culture Collection of Charles University Prague, Czech Republic. There are no paratypes designated for the species. **Type host**: *Culicoides truncorum*, Diptera, Ceratopogonidae. **Locality:** Moravia, Czech Republic. **Morphology:** Markedly polymorphic with very large promastigotes among some rather small forms. Original measurements are in Zídková et al. (2010). No opisthomastigotes were reported. **Diagnosis:** SSU rRNA and gGAPDH sequences (Table 1) deposited in Genbank by Zídková et al. (2010).

### 1.6. ***Herpetomonas mariadeanei*** Yoshida, Freymuller and Wallace, 1978

**Type material:** hapantotype, culture ATCC 30708 from which TCC004E, deposited at TCC-USP, is the original. **Type host**: *Muscina stabulans*, Diptera, Muscidae. **Locality**: São Paulo, Brazil. **Morphology**: Promastigotes and endomastigotes (Fig. 5q, r). Culture and Giemsa-stained glass smears are deposited at TCC-USP. Measurements in Yoshida et al. (1978).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**Diagnosis:** SSU rRNA (JQ359714), gGAPDH (JQ359741) and ITS1 rDNA (JQ406956-58) sequences deposited in Genbank.

## 2. New Species of *Herpetomonas*

### 2.1. *Herpetomonas isaaci* Teixeira and Camargo n. sp.

**Type material:** hapantotype, culture TCC266E; paratypes, cultures TCC276E, 270, 278E, 1533, 1699, 1724, 1766, 1767, 1852, 1881, 1882, 1885, 1941, 1944, 2026 and 2069, whose hosts and locality of collection are in Table 1. Cultures and Giemsa-stained glass smears are deposited at TCC-USP. **Type host**: *Musca domestica* (Diptera, Muscidae). **Habitat***:* Intestine. **Locality***:* Rolândia, Paraná, Brazil, S26°20′ W 51°26′. **Morphology**: Promastigotes often with rounded posterior ends and relatively wide cellular bodies. Opisthomastigotes resembled opisthomorphs (Fig. 5c, g). Promastigotes body length, 6.4 – 11.9 μm (8.8 ± 1.4 μm), body width 2.0 – 3.0 μm (2.6 ± 0.3 μm) and length of free flagellum 5.2 – 18 μm (9.5 ± 3.0 μm). **Diagnosis**: Based on DNA sequences deposited in Genbank: SSU rRNA (JQ359720), gGAPDH (JQ359745) and ITS rDNA (JQ406965-67). **Etymology***:* The name is given in honour of Prof. Isaac Roitman, a Brazilian Protozoologist with a significant contribution in the field of insect trypanosomatids.

### 2.2. *Herpetomonas puellarum* Teixeira and Camargo n. sp.

**Type material:** hapantotype, culture TCC1745; paratypes, cultures TCC247E and 254E, whose hosts and locality of collection are in Table 1. Cultures and Giemsa-stained glass smears are deposited at the TCC-USP. **Type host**: *Chrysomya putoria* (Diptera: Calliphoridae). **Habitat***:* Intestine. **Locality**: Cufada, Buba, Guinea Bissau, N43°03′W15°02′. **Morphology**: Promastigotes may display truncated posterior ends. Some flagellates display vase-like, funnelled anterior extremities resembling choanomastigotes. Their opisthomastigotes are shorter, and may resemble the opisthomorphs of symbiont-harbouring flagellates (Fig. 5a, e), but contrary to those, they are non-multiplying forms and remain rare in cultures. Measurements of promastigotes are: body length 5.6 – 8.4 μm (7.0 ± 0.8 μm); body width 2.6 – 1.9 μm (2.3 ± 0.3 μm); free flagellum 4.9 – 9.0 μm (6.6 ± 1.5 μm). **Diagnosis**: Based on DNA sequences deposited in Genbank: SSU rRNA (JQ359723), gGAPDH (JQ359734) and ITS rDNA (JQ406971-73). **Etymology***:* the name *puellarum*, Latin Genitive, "of girls", is given to do homage to the little girls who joyfully encouraged our collection of flies at their school grounds in Buba, Guinea Bissau, Africa.

### 2.3. *Herpetomonas elegans* Teixeira and Camargo n. sp.

**Type material***:* hapantotype, culture TCC1733; paratypes, cultures TCC1727 and 1848, whose host and locality of collection are in Table 1. Cultures and Giemsa-stained glass smears are deposited at the TCC-USP. **Type host***: Ornidia obesa* (Diptera: Syrphidae). **Habitat***:* Intestine. **Locality***:* Monte Negro, Rondônia, Brazil, S10°15′ W63°17′. **Morphology**: Promastigotes slender and rather small but homogeneous. Elongated opisthomastigotes are common (>15%) (Fig. 5d, h). Measurements of promastigotes: body length 4.6 – 9.4 μm (7.1 ± 1.1 μm); body width 1.2 – 2.6 μm (1.9 ± 0.3 μm); free flagellum 4.4 – 16.3 (8.6 ± 3.0 μm). **Diagnosis**: Based on DNA sequences deposited in Genbank: SSU rRNA (JQ359725), gGAPDH (JQ359735) and ITS rDNA (JQ406977-79). **Etymology**: the name *elegans*, Latin adjective, "elegant", is given because of the flagellate harmonious simplicity.

### 2.4. *Herpetomonas modestus* Teixeira and Camargo n. sp.

**Type material:** hapantotype, culture TCC 1444; paratypes: cultures TCC 1943 and 2049, whose host and locality of collection are in Table 1. Cultures and Giemsa-stained glass smears are deposited at the deposited at TCC-USP. **Type host***: Chrysomyia megacephala* (Diptera, Calliphoridae). **Habitat***:* intestine. **Locality**: Campo Grande, Mato Grosso do Sul, Brazil, S19°54′ W55°16′″. **Morphology***:* Polymorphic small promastigotes, sometimes exhibiting a long and thin posterior extremity and very often a truncated anterior end. Opisthomastigotes are small and sturdy (Fig. 5l, j). Measurements of promastigotes are: body length 7.6 – 15 μm (10.6 ± 2.0 μm); body width 1.7 – 2.8 μm (2.2 ± 0.3 μm); free flagellum 10.7 – 24.1 μm (13.5 ± 2.7 μm). **Diagnosis**: Based on DNA sequences deposited at GenBank: SSU rRNA (JQ359726), gGAPDH (JQ359736) and ITS rDNA (JQ406980-82). **Etymology**: the name *modestus*, Latin nominative, "modest", is given because of its unpretentious morphology in culture.

### 2.5. *Herpetomonas wanderleyi* Teixeira and Camargo n. sp.

**Type material**: hapantotype, culture TCC 1982 and Giemsa-stained glass smears are deposited at the TCC-USP. **Type host**: *Chocliomyia macellaria* (Diptera, Calliphoridae). **Habitat***:* intestine. **Locality***:* Miranda, Mato Grosso do Sul, Brazil, S20°15′ W80°22′. **Morphology***:* Very large promastigotes with marked polymorphism, including very long and twisted promastigotes as those described by Roubaud (1908) in *L. mirabilis (= H. mirabilis)* and filiform flagellates, which are the

**Figure 7.** Ultrastructural organization by transmission electron microscopy of new and reference-species of *Herpetomonas*: *H. muscarum* (**a, b, d**); *H. modestus* (**c**); *H. isaaci* (**e**); *H. samuelpessoai* (**g**); *H. puellarum* (**f, i**); *H. mirabilis* (**d, h**); *H. wanderleyi* (**j, k**). Longitudinal sections of promastigotes showing very large (**a**), intermediary (**e**) and small (**i**) body length. Highly developed tubules of the spongiome (**c, f, g**) draining to the contractile vacuole attached to the membrane of the flagellar pocket (**f, g**). Flagellar pockets (**c, e-g**). Paraflagellar rod (PFR) structure of the flagellum: longitudinal section of proximal domain inside the flagellar pocket (**b)** and distal domain showing the typical lattice-like arrangement of the PFR filaments **(j**); transversal sections (**d, f, k**) of the PFR adhered to the axonemal microtubules. Mitochondria (**a, b, d–f, l**) exhibiting exuberant cristae (arrows). Rod-shaped kinetoplast (**b, e, g-i, l**). N, nucleus; M, mitochondrion; K, kinetoplast, F, flagellum; Fp, flagellar pocket; Sp, spongiome; Cv, contractile vacuole; PFR, paraflagellar rod; Ac, acidocalcisomes; G, glycosomes; GO, Golgi Complex; Bb, basal body; mt, microtubules. Bars = 0.5 μm. Arrowheads (c) point to hemidesmosomes. Bars = 0.5 μm.

longest promastigotes ever described. No opisthomastigotes were present (Fig. 6a - e). Measurements: promastigotes body length 13.4 – 66.2 μm (31.1 ± 13.6 μm); body width 1.3 – 5.5 μm (2.5 ± 0.9 μm); free flagellum 10.3 – 25.8 μm (16 ± 4.7 μm). **Diagnosis:** Based on DNA sequences deposited in Genbank: SSU rRNA (JQ359730), gGAPDH (JQ359740) and ITS rDNA (JQ417274). **Etymology***:* the name is given in honor of Prof. Wanderley de Souza, a leading Brazilian Protozoologist with considerable contribution to the cell biology of trypanosomatids in general.

### 3. New combinations

### 3.1. ***Herpetomonas samueli*** (Carvalho, 1973) Teixeira and Camargo n. comb.

This species corresponds to former *Leptomonas samueli* isolated by Carvalho (1973) from the digestive tube of *Zelus leucogrammus.* **Type material:** hapantotype, culture ATCC 30971 of which culture TCC 003E, deposited at the TCC-USP, is a duplicate. Giemsa-stained glass smears are deposited at TCC-USP. **Type host**: *Zelus leucogrammus*, Hemiptera Reduvidae. **Locality**: Goiania, Goiás, Brazil. **Morphology**: Promastigote body length, 12.8 – 20.4 μm (16 ± 2 μm), body width 1.1 – 1.8 μm (1.5 ± 0.2 μm) and flagellum length 9.5 – 23.6 μm (17.4 ± 0.5 μm). Opisthomastigotes were not reported in the original descriptions of the species, probably, due to their very low percentage in cultures (<0.1%). **Diagnosis**: Sequences deposited in Genbank from SSU rRNA (JQ359722), gGAPDH (JQ359742) and ITS rDNA (JQ406986-88). **Comments:** The SSU rRNA and gGAPDH sequences from *H. samueli* determined in this study and corresponding sequences from *L. lactosovorans* in Genbank (SSU rRNA: EU079122 and gGAPDH: EU076602) are nearly identical. As already suggested (Maslov et al. 2010; Merzlyak et al. 2001; Teixeira et al. 1997; Zídková et al. 2010), *L. lactosovorans* is possibly a species of *Herpetomonas;* in this case, it would be a synonymous to *L. samueli.* However, these species have metabolic and morphological differences, hence, additional data, preferentially from a new sample, are necessary for a definite taxonomy of *L. lactosovorans*.

### 3.2. ***Herpetomonas costoris*** (Wallace et al.1965) Teixeira and Camargo n. comb.

This species corresponds to former *Leptomonas costoris* isolated from the water-strider, *Gerris comatus* (Wallace et al. 1965). **Type material:** hapantotype, culture ATCC 30262 of which culture TCC 019E, deposited at TCC-USP, is a replicate. Giemsa-stained smears are deposited at TCC-USP. **Type host**: *Gerris comatus*, Hemiptera Gerridae. **Locality**: Minnetonka, Minnesota, USA. **Morphology**: Promastigotes are polymorphic with one or two twists along the body and, in general, a long and pointed posterior end (Fig. 5m, n). Opisthomastigotes were not detected. Promastigote body length, 13 – 19.5 μm (16.1 ± 2.6 μm), body width, 2.1 – 3.5 μm (2.9 ± 0.5 μm) and flagellum length 15.3 – 31.7 μm (23.3 ± 5.9 μm). **Diagnosis:** DNA sequences from SSU rRNA (JQ359728), gGAPDH (JQ359738) and ITS rDNA (JQ406983-85) in Genbank.

### 4. Trypanosomatids of Other Genera Formerly Classified as *Herpetomonas*

Some species formerly classified as *Herpetomonas* were not positioned in the *Herpetomonas* clade in any inferred phylogenetic trees and should, therefore, be reclassified in other genera. They are: a) *Herpetomonas roitmani* which groups with trypanosomatids carrying symbionts and has already been included in the genus *Angomonas* as a synonym of *A. deanei* (Teixeira et al. 2011); b) *Herpetomonas mcgheei* that has already been renamed as a species of the genus *Phytomonas* on the basis on SL sequences (Camargo 1998; Serrano et al. 1999; Teixeira et al. 1996); c) *Herpetomonas dedonderi*, which culture, even cloned, exhibit promastigotes that differentiate to choanomastigotes, and does not fit in the genus *Crithidia* nor in *Herpetomonas* according to molecular data (Camargo et al. 1992, Teixeira et al. 1997); d) *Herpetomonas anglusteri* was found to be more related to *C. fasciculata* than to *Herpetomonas* using ribosomal markers (Teixeira et al. 1997). All these data are being currently supported by SSU rRNA analyses (unpublished data).

## Discussion

The Trypanosomatidae consist of clonally propagated populations (Tibayrenc and Ayala 1996). Although capable of genetic recombination (Gaunt et al. 2003), trypanosomatids do not benefit from sex to validate their taxonomy. Until recently, genus and species identification of trypanosomatids relied on their morphology, behaviour and biochemical characteristics as well as on their host and geographic origin. In the case of trypanosomatids with more than one kind of host, particularly those of *Trypanosoma*, diagnosis was facilitated by life cycles involving hematophagous invertebrates, sylvatic or domestic animals and, in some cases, humans.

Most species of trypanosomatids parasitising exclusively insects are morphologically inconspicuous. Geographic and host origin are of limited taxonomic value for these parasites. In the past, when host origin was thought to be stringent at the level of species, insect trypanosomatids might be simply defined by the host origin criterion (Wallace 1966; Wallace et al. 1983) and many species have been designated accordingly. However, the uncovering of generalist and cosmopolitan trypanosomatids plus the fact that an insect species may harbour more than one trypanosomatid species weakened the validity of these criteria. For example, we found that *Musca domestica* harbours not only *H. muscarum* but also *H. samuelpessoai*, *H. isaaci*, *H. puellarum* and *H. elegans*. Some of the host *M. domestica* are from the USA, whereas others are from various parts of Brazil. Inversely, *H. samuelpessoai* colonises different insect species from Brazil and Africa, such as *M. domestica, Fannia canicularis, Sarcophaga* sp., the predatory hemipteran *Zelus leucogrammus* and blowflies of *Lucilia* and *Chrysomyia.* Regarding geographical origin, *M.*

*domestica* is a cosmopolitan and synanthropic fly, which apparently colonised the American continent after Columbus (Cummings and Krafsur 2005). We demonstrated that *H. muscarum* isolated from *M. domestica* in the USA was identical to several isolates from diverse regions of Brazil. A similar scenario was presented by *H. samuelpessoai* from blowflies, a cosmopolitan species of synanthropic flies in the American and African continents. Cosmopolitanism has also been reported for trypanosomatids from hemipterans (Votýpka et al. 2012).

In this study, we showed that some *Herpetomonas* species are generalist trypanosomatids from flies and appear to be as cosmopolitan as their hosts, corroborating that geographical or host origin cannot be used as absolute taxonomical criteria for genus and species identification of insect trypanosomatids. However, this lack of specificity is not equivalent to stating that insect trypanosomatids do not have host preferences and it is conceivable that some species may be restricted to a particular host and/or place. In fact, our data showed a marked preference of *Herpetomonas* species for dipterans. Out of 527 trypanosomatids from hemipterans and dipterans examined, *Herpetomonas* occurred in 47 dipterans and three predator hemipterans, which usually feed on dipterans. The origin of two isolates from plants, spurge and tomatoes, is questionable. We believe that the plants may have been contaminated by insect faeces. Infection of plants with insect trypanosomatids is rather common (Batistoti et al. 2001; Camargo 1998; Serrano et al. 1999; Teixeira et al. 1996) and tomatoes are particularly susceptible to them (Conchon et al. 1989).

With respect to the morphological criterion, the scenario is somewhat more complicated. The intra-specific polymorphism of *Herpetomonas* complicates the diagnosis of species based on morphotypes. Forms that we thought to be specific of a given species or genera were also found in phylogenetically distinct species. Although not in profusion, vase-like flagellates identifiable as choanomastigotes and considered to be characteristic of *Crithidia* (Wallace 1966) may be observed in cultures of many species of *Herpetomonas* and other trypanosomatid genera (Teixeira et al. 1997, 2011). Similarly, the long, twisted cellular bodies present in some *Herpetomonas* species were thought to be diagnostic of *Phytomonas* (Camargo 1998).

Molecular data have evidenced many flaws in the current host and morphology-based taxonomy of the genus *Herpetomonas*, which by previous definition includes promastigotes and opisthomastigotes in the insect trypanosomatids' life cycles. Many species of *Herpetomonas* thus identified lack opisthomastigotes and have an unorthodox morphology distinct from the classical promastigotes of *H. muscarum*. Regarding morphological diversity, species of *Herpetomonas* exhibit morphotypes of other trypanosomatid genera: promastigotes (genera *Leptomonas* and *Leishmania*), choanomastigotes (*Crithidia*) and twisted promastigotes (*Phytomonas*). In fact, conflict between morphology and phylogeny has been reported in many instances among trypanosomatids. Yurchenko et al. (2008) referred to promastigotes in a presumed species of *Leptomonas* that are phylogenetically closer to the genus *Crithidia.* Teixeira et al. (2011) disclosed symbiont-harbouring trypanosomatids of the genus *Angomonas* previously classified as *Herpetomonas* or *Crithidia*.

In the present work, the genus *Herpetomonas* was redefined by molecular phylogenetic analyses based on independent and concatenated SSU rRNA and gGAPDH genes as a clade well supported in all phylogenetic analyses. The genus *Herpetomonas* presents high inter-specific genetic variability and species boundaries within this genus were delimited by sequence divergences of SSU rRNA, gGAPDH and ITS1 rDNA, besides species-specific putative secondary structures evidenced for all species examined. The application of the analyses of both ITS1 primary and secondary structures successfully complemented the results obtained using SSU rRNA and gGAPDH genes. To our knowledge, this is the first time that comparison of putative secondary structures was used to address phylogenetic and taxonomical questions in trypanosomatids. This approach has been widely and successfully employed for phylogenetic and taxonomic studies of eukaryotes, improving the comparisons between nucleotide sequences and, hence, phylogenetic inferences. In addition, comparison of ITS base changes has been successfully used to distinguish species of various protists (Letsch et al. 2010; Miao et al. 2008; Ponce-Gordo et al. 2011; Schultz and Wolf 2009; Sun et al. 2010; Thornhill and Lord 2010). Results from this study demonstrated that a simple visual inspection of secondary structure of ITS1 regions is enough to identify all the 11 species of *Herpetomonas* investigated.

Altogether, data from the combined morphological and phylogenetic approaches employed in this study revealed that the morphological and molecular diversity within *Herpetomonas*, as defined in this study, are the highest among all

phylogenetically validated genera of monoxeneous insect trypanosomatids. The phylogenetic data presented in this paper uncovered that some flagellates, classified as *Herpetomonas* by strict morphological criteria, should be classified in different genera according to phylogeny or, inversely, that some flagellates classified in the genera *Leptomonas* or *Phytomonas* should be reclassified as *Herpetomonas*.

It is becoming increasingly evident that molecular data will promote a full reappraisal of the taxonomy of insect trypanosomatids. We believe that the present disassociation of the genus *Herpetomonas* from its former morphological definition is an important step in that direction. Phylogenetic analyses based on SSU rRNA and gGAPDH sequences indicate that the genus *Herpetomonas* is monophyletic and constituted by solitary species alongside species-congregating isolates from diverse hosts and geographical origins. The taxa we have nominated can be reviewed at any time in the presence of new evidence. The gradual improvement of classifications is part of the dynamics of taxonomy. It will not be surprising whether *H. mariadeanei* and, perhaps, strongly divergent species, such as *H. wanderleyi* and *H. mirabilis*, are accommodated in other taxa after larger samples and new molecular markers become examined. However, the data obtained so far recommend that these species remain in the genus *Herpetomonas* until analysis of a larger sample, using additional molecular makers, indicates otherwise.

The recognition of trypanosomatid species remains a problem similarly to asexual protozoans in general. Our data favour the separation of trypanosomatid species based on ancestor-descendent lineages rather than by phenotypic, geographic or host origin criteria. However, in addition to phylogenetic data, morphological, behavioural and bio-geographical information plus common sense are valuable for trypanosomatid species designation. We do not expect the present classification to be definitive, but we hope that the proposed taxa may help to elaborate on the criteria needed for a better and more natural classification of the Trypanosomatidae. The phylogenetic data gathered in this study may serve as a framework for the future reconnaissance of a member of *Herpetomonas* among trypanosomatids in general.

## Methods

**Isolation, culture and preservation of the trypanosomatids:** We have examined a total of 527 trypanosomatids isolated from flies and a few from other insects deposited at our Trypanosomatid Culture collection (TCC-USP) of the University of São Paulo, São Paulo, Brazil. The collection and dissection of flies and the culture of isolates were done as previously described (Sbravate et al. 1989; Teixeira et al. 1997, 2011) and information about hosts and localities of insect collection are in Table 1. Flagellates were preliminarily examined by light microscopy in fresh preparations of insect guts for subsequent culturing of positive samples in TC 100 medium supplemented with 2-5% SFB at 25-28 °C. After the removal of contaminating fungi and bacteria all cultures have been cloned and re-cloned as described before (Sbravate et al. 1989). Giemsa-stained smears from insect guts are cryopreserved in liquid nitrogen at the TCC-USP. Cultures of some reference trypanosomatids are also deposited at the American Type Culture Collection (ATCC).

**Sequencing and data analyses of V7V8 SSU rRNA, SSU rDNA and gGAPDH genes:** Total DNA was extracted from cultured flagellates using the traditional phenol-chloroform method. PCR amplification of the V7V8 SSU rRNA region (~750-800 bp) was performed using the following primers: 609 F - 5′CACCCGCGGTAATTCCAGC3′ and 706 R - 5′CTGAGACTGTAACCTCAA3′. Reaction mixtures (50ul) containing ~100 ng of DNA, 100 ng of each primer, 200 μM of each dNTP, 1.5 mM of $MgCl_2$ and 2.5 U of Taq DNA polymerase were submitted to 30 cycles as follows: 1 min at 94 °C, 2 min at 48 °C and 2 min at 72 °C (with an initial cycle of 3 min at 94 °C and a final cycle of 10 min at 72 °C). Whole SSU rRNA (~2 Kb) sequences were amplified using the KRD5 - 5′GATCTGGTTGATTCTGCCAGTAG3′ and KRD3 - 5′GATCCAGCTGCAGGTTCACCTAC3′ primers under the same PCR conditions described above for V7V8 SSU rRNA region, except for the annealing temperature of 55 °C (Ferreira et al. 2008; Teixeira et al. 2011). Amplification of whole gGAPDH genes (~800 bp) was done using GAPTRY-mod F - 5′GGBCGCATGGTSTTCCAG3′ and GAPTRYr R - 5′CCCCACTCGTTRTCRTACC3′ primers under the same PCR conditions described above for the other genes at the annealing temperature of 55 °C (Viola et al. 2008; Teixeira et al. 2011).

PCR amplified DNA from V7V8 SSU rRNA, SSU rRNA and gGAPDH genes were eletrophoresed in a 2.0% agarose gel and stained with ethidium bromide, and the amplified fragments from 2-3 independent PCR reactions were purified from agarose gels, sequenced and deposited in GenBank (Table 1). Sequences were aligned using CLUSTALX (Thompson et al. 1997) and manually refined. Three alignments were generated and analyzed using Parsimony (P), Maximum Likelihood (ML) and Bayesian inference (BI) analyses: A1, consisting of the V7V8 SSU rRNA of *Herpetomonas* spp. (766 characters); A2, sequences of SSU rRNA, without regions of ambiguous alignment, from trypanosomatids representing all genera (1.932 characters); A3, containing the gGAPDH sequences from trypanosomatids representing all genera (864 characters); and A4, consisting of concatenated SSU rRNA and gGAPDH sequences from all genera of trypanosomatids (2.796 characters). All alignments used in this study are available from the authors upon request.

The parsimony analyses were carried out using PAUP* 4.0b10 (Swofford 2002). For the tree search and bootstrap we used 500 random sequence addition replicates with TBR. The ML analyses were performed using RAxML 7.2.6 (Stamatakis 2006) as described previously (Ferreira et al. 2008; Viola et al. 2009). The Bayesian analyses employed GTR + Γ+ I model with the tree parameters estimated by likelihood (proportion of invariable sites equal to 0.512 and the gamma distribution shape parameter for variable sites equal to 0.647) selected in MrModeltest 2.3 (Nylander 2004) using the Akaike information criterion

(AIC). In MrBayes 3.1.2 we run 1.000.000 generations with the remaining parameters set to default. The burn-in was set to 25% in all analyses and stationary was evaluated monitoring post-burn-in values of the mean log-likelihood.

**Analysis of ITS1 rDNA sequences and secondary structure models:** PCR amplification of the ITS1 rDNA was performed using the primers IR1 - 5′GCTGTAGGTGAACCTGCAGCAGCTGGATCATT3′ and 5.8R - 5′GGAAGCCAAGTCATCCATC3′. PCR reactions were performed using a mixture of ∼100 ng of DNA templates, 100ng of each primer, 200 μM of each dNTP, 1.5 mM of $MgCl_2$ and 2.5 U of Taq DNA polymerase submitted to 30 cycles as follows: 1 min at 94 °C, 2 min at 55 °C and 2 min at 72 °C (with an initial cycle of 3 min at 94 °C and a final cycle of 10 min at 72 °C). The PCR-amplified products were purified from 2.0% agarose gels, cloned, and at least 3 clones from each flagellate were sequenced as described previously (Maia da Silva et al. 2004).

Alignment of ITS1 rDNA sequences from *Herpetomonas* spp. obtained using Clustal X 2.1 and structural information (Letsch et al. 2010) was refined manually. ITS1 rDNA sequences of other trypanosomatid genera were not included in the analysis due to unreliable alignments. Phylogenetic inferences were assessed by the Maximum Likelihood (ML) method as described above. Predicted secondary structures were inferred for 11 species of *Herpetomonas*, including more than one isolate for species, using the Unafold package (Markham and Zuker 2008). Energy minimization of paired and unpaired regions was the approach employed to infer ITS1 secondary structures (thermodynamically predicted models) of *Herpetomonas* spp using 25 °C and 37 °C folding temperatures (Markham and Zuker 2008). Highly similar secondary structure models were recovered for each species at both temperatures. The secondary structures were visualized using the RnaViz program (DeRijk et al. 2003).

**Morphological analysis by light and electron microscopy:** Smears of logarithmic phase cultures were fixed in methanol and stained with Giemsa. Flagellates were photographed with a digital camera and ∼ 30-40 flagellates from each culture were measured with LSM Image Browser 4.0.0.157 (Zeiss). For scanning electron microscopy SEM, cultures were fixed in 2.5% (v/v) glutaraldehyde plus 1% (w/v) paraformaldehyde in 0.1 M cacodylate buffer (pH 7.2), adhered to 0.1% (v/v) poly-L-lysine-coated coverslips, post-fixed in 1% (w/v) $OsO_4$ in 0.1 M cacodylate buffer for 30 min, dehydrated in an ascending ethanol series, critical point-dried with $CO_2$ in a Balzers CPD 030, sputtered with gold in a Sputter Coater SCD 050 Balzers), and observed using a DSM 940 Zeiss Digital Scanning Microscope. For TEM, cells were fixed as described above, post-fixed in 1% (w/v) $OsO_4$ in 0.1 M cacodylate buffer for 60 min, stained overnight with 0,5% uranyl acetate at 4 °C, dehydrated in ethanol and embedded in Spurr's resin as described before (Teixeira et al. 2011; Viola et al. 2009). Ultrathin sections were stained with uranyl acetate and lead citrate and examined using a Jeol 100 CX Electron Microscope.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.protis.2012.06.001.

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