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REVIEWS

Review

Trypanosomatids Are Much More than Just Trypanosomes: Clues from the Expanded Family Tree

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Trypanosomes and leishmanias are widely known parasites of humans. However, they are just two out of several phylogenetic lineages that constitute the family Trypanosomatidae. Although dixeny – the ability to infect two hosts – is a derived trait of vertebrate-infecting parasites, the majority of trypanosomatids are monoxenous. Like their common ancestor, the monoxenous Trypanosomatidae are mostly parasites or commensals of insects. This review covers recent advances in the study of insect trypanosomatids, highlighting their diversity as well as genetic, morphological and biochemical complexity, which, until recently, was underappreciated. The investigation of insect trypanosomatids is providing an important foundation for understanding the origin and evolution of parasitism, including colonization of vertebrates and the appearance of human pathogens.

One Host or Two? Lifestyles of the Trypanosomatidae

All members of the family Trypanosomatidae are obligatory parasites and include the iconic pathogens responsible for African sleeping sickness, Chagas' disease and leishmaniases. Such untold suffering justifies the intense research on their causative agents – *Trypanosoma brucei*, *T. cruzi* and *Leishmania* spp. These parasites are transmitted to a vertebrate host by an invertebrate vector, mostly an insect, but with dramatic differences in their survival strategies and life cycles. For example, *T. brucei* and related salivarian trypanosomes undergo a complex development in a tsetse fly, resulting in the production of infective flagellates in the salivary glands. Propagation in the vertebrate occurs in the bloodstream, with antigenic variation protecting the trypanosome population from the host's immune response. On the other hand, *T. cruzi* is transmitted via the feces of an infected reduviid bug. A chronic infection of the vertebrate host is maintained by intracellular propagation of parasites in the smooth muscles and other host tissues. The genus *Leishmania* is only distantly related to trypanosomes and shows yet another set of dixenous adaptations. Leishmanias are transmitted to mammals by sand flies, and they evade elimination from the bloodstream by propagation in macrophages substantially remodeled to suit the parasite's needs.

It is now clear that dixenous parasitism has independently evolved several times from the monoxenous (= infecting a single host, usually an invertebrate) ancestors. Therefore, an entire range of questions regarding the origin, evolution, and many aspects of cell and molecular biology of these pathogens can be answered only by studying their relatives that are non-pathogenic to vertebrates [1]. These monoxenous parasites have long remained in the backwaters of trypanosomatid research, although they represent the bulk of the family and, thus,

Highlights

Dixenous trypanosomatids, such as the human *Trypanosoma* parasites, infect both insects and vertebrates. Yet phylogenetic analyses have revealed that these are the exception, and that insect-infecting monoxenous lineages are both abundant and diverse.

Globally, over 10% of true bugs and flies are infected with monoxenous trypanosomatids, whereas other insect groups are infected much less frequently. Some trypanosomatids are confined to a single host species, whereas others parasitize a wide spectrum of hosts.

Many trypanosomatids are themselves infected with viruses and bacteria that have been acquired from insects, terrestrial invertebrates, and fungi. At least two lineages contain bacteria; these endosymbiotic events occurred independently and evolved differently.

Genomes and transcriptomes of monoxenous trypanosomatids will bring new insight into the origins of parasitism and how trypanosomes and leishmanias evolved their capacity to infect humans and other vertebrates.

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define it in many ways. These organisms still conceal a large volume of information resembling the underwater part of the proverbial iceberg, which threatens to sink any evolutionary theory that does not take its existence into account. The issue of utmost importance is to uncover the true dimensions of trypanosomatid diversity, especially with respect to discovery of the major phylogenetic lineages, and identification of the closest relatives to the dixenous parasites. Evolutionary scenarios leading to the more advanced dixenous life strategy would then be reconstructed with comparative genomics and phylogenomics.

Phylogeny and Diversity

The relationships between the dixenous (usually having one vertebrate or plant and one invertebrate host) flagellates and their monoxenous relatives have been debated for decades [2]. Current phylogenies strongly argue for multiple and independent origins of the dixenous life style [3], although the still fragmentary nature of the phylogenetic trees renders any comprehensive evolutionary scenario a task for the future. Nevertheless, the origin of dixenous *Leishmania* from monoxenous trypanosomatids has been well supported by recent works (Figure 1) [4,5].

Since only a small fraction of potential host species in a limited number of countries has been sampled (see Figure S1 in the supplemental information online), estimates of the global biodiversity of monoxenous trypanosomatids would be premature. Indeed, the species accumulation rate has not shown signs of slowing down, indicating that the known segment of the biodiversity is relatively small. The tally necessarily excludes a score of 'old' species [6], described on the basis of the now refuted 'one host – one parasite' paradigm (Figure S2). Current taxonomic practice includes molecular barcoding with 18S rRNA, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and/or spliced leader (SL) RNA gene sequences in combination with additional taxonomically relevant information such as the organism's morphology and life history [7]. A formal species description requires the availability of an *in vitro* culture, which has turned out to be a serious impediment, since many species have proven to be fastidious or even uncultivable with available media. In addition, for hosts with mixed trypanosomatid infections, cultivation results in the selection of the fastest growing species, therefore misrepresenting natural populations of parasites [8]. An alternative is a culture-independent approach, which includes identification and molecular barcoding of parasites directly in the infected host [9]. The taxonomic entities thereby discovered are referred to as typing units (TUs) and represent proxies of species that lack detailed morphological descriptions but still retain some additional characteristics, such as host identity, general morphotype, and localization in the host. At present there are about 300 monoxenous trypanosomatid TUs (Figures S1 and S2), which is, without any doubt, only a minor segment of their true diversity.

Most of these TUs have been found in insects from the orders Heteroptera (true bugs) and Diptera (flies) (Figure S2). This situation likely reflects the actual preference of monoxenous trypanosomatids for these host groups, which are best suited for transmission, the likely bottleneck stage in their life cycles. Unlike dixenous parasites, which can be stably maintained after establishing a reservoir in vertebrates, their monoxenous kin are critically dependent on the ability of infected hosts to pass on the parasites among themselves (Box 1). These opportunities are provided by the natural histories of social or predatory insects. About 200 TUs, representing ~70% of the total, have been found in 28 heteropteran families, two of which display the highest prevalence levels and the largest diversity ranges: (i) the insectivorous Reduviidae, with predation as the most likely mode of transmission; (ii) the social Pyrrhocoridae, with transmission by coprophagy, necrophagy, and contamination (Figure S3). In dipterans, most of the identified 50 TUs have been found in the suborder Brachycera, known for their

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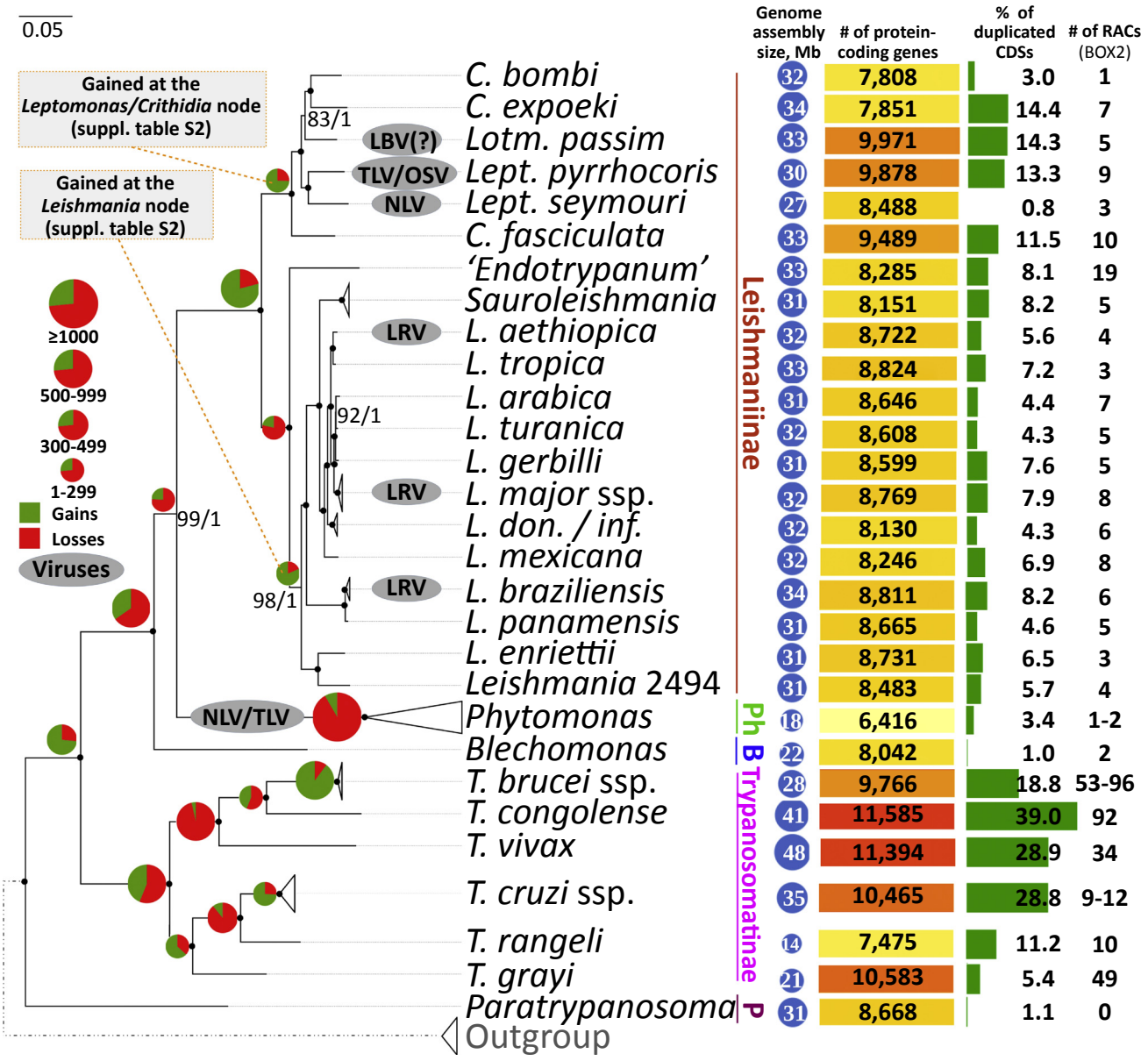


Trends in Parasitology

Figure 1. Phylogeny, Viruses, Genomes and Their Selected Features. A phylogenetic tree based on the alignment of 64 conserved proteins. Parabodonid *Trypanoplasma borreli* and eubodonid *Bodo saltans* served as outgroups. The tree was constructed with IQ-TREE v. 1.5.3 using the LG+F+I+G4 model and 1000 bootstrap replicates and PhyloBayes-MPI v. 1.7b under the GTR-CAT model with four gamma categories run for ~10 000 generations. Only bootstrap support values lower than 100% and posterior probabilities lower than 1 are shown. The scale bar represents 0.05 substitutions per site. The composition of the clades can be found in Table S1 in the supplemental information online. The haploid genome assembly size for each species is represented by blue circles with an area proportional to the given values. Numbers of protein-coding genes are shown using a color-scale. Green data bars demonstrate coding sequence (CDS) duplication levels. Various trypanosomatid viruses are shown in gray ovals. OGs gains and losses were inferred with the COUNT software. Abbreviations: LBV, *Leishbunyavirus*; LRV, *Leishmaniavirus*; NLV, Narna-like virus; OSV, ostravirus; TLV, Tombus-like virus; RACs, receptor adenylate cyclases; OGs, orthologous groups of proteins; B, Blechomonadinae; P, Paratrypanosomatinae; Ph, Phytomonadinae.

Box 1. Relationship with the Insect Hosts

A majority of monoxenous trypanosomatids parasitizes the midgut (Figure I). Especially in dipterans, the peritrophic matrix completely separates enterocytes from the food mass, also becoming a major barrier for the flagellates. But many species (e.g., *Blastocrithidia triatomae*) escape from the endoperitrophic space, weave their modified flagella among microvilli, and attach to the surface of the host's intestinal epithelium [59]. Moreover, other species (e.g., *Blastocrithidia raabei*, *Crithidia fasciculata*, and *Leptomonas pyrrhocoris*) migrate through the midgut wall into the body cavity. A substantial fraction of trypanosomatids (e.g., dixenous *T. cruzi* and the subgenus *Sauroleishmania*; monoxenous genera *Herpetomonas* and *Crithidia*) passes through the midgut into the hindgut and rectum. Parasites attach to the hindgut cuticular lining usually via hemidesmosomes, by mechanisms similar to those used by leishmanias and trypanosomes at the anterior intestine on the stomodeal/pharyngeal valve [60] or by *Herpetomonas nabicula* [61]. In the hindgut, the surface of rectal glands is preferably colonized, with the flagellates forming huge multilayered clusters. Finally, the Malpighian tubules are rarely colonized (e.g., *B. triatomae*, *L. pyrrhocoris*, *Herpetomonas ztiplika*) (Figure I).

According to the effect on the insect hosts, three pathogenicity classes can be recognized: subpathogenic, intermediate, and pathogenic [62]. The first, most common type does not alter the host's life span and fitness, but can have an adverse effect under stress conditions, while pathogens seriously affect their host even under normal condition. Most dixenous species of biomedical importance are intermediate-level or subpathogenic to their insect hosts. Following ingestion, most trypanosomatids pass throughout the cuticle-coated foregut into the midgut, with just a few *Trypanosoma* spp. developing in the proboscis and foregut. While most *Leishmania* and some *Trypanosoma* spp. may damage the stomodeal valve and affect vector feeding efficiency [60], there is no strong influence by *T. cruzi* on its triatomine hosts in the absence of stress [63].

Generally, most monoxenous trypanosomatids have no or very little effect on their hosts, behaving as commensals [64]. However, there are a few notable exceptions, such as the highly pathogenic *B. triatomae*, which is transmitted directly by cannibalism and/or coprophagy, and forms a cyst-like stage that facilitates its survival outside the host [62]. During infection, *Jaenimonas drosophilae* induces an immune response in its *Drosophila* spp. hosts [11]. The impact of trypanosomatids on host populations is exemplified by bee parasites. The newly described *Lotmaria passim* is likely responsible for (most) pathologies so far attributed to *Crithidia mellificae*, and likely the predominant trypanosomatid in honey bees worldwide [65,66]. Both parasites have been linked to increased colony mortalities in Europe and the USA. The related *Crithidia bombi* is known to have serious effects on bumble bee behavior, feeding strategy, health, and colony fitness [67].

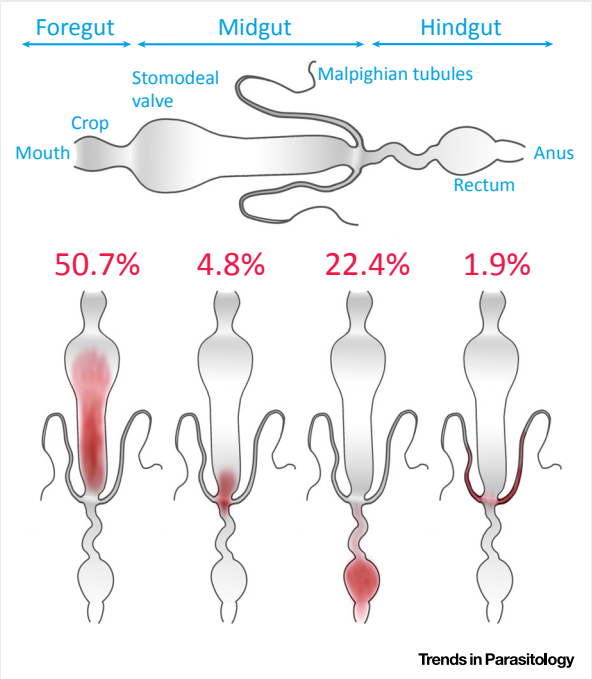


Figure I. Location of the Infection in the Heteropteran and Dipteran Hosts. Percentages are based on 1144 dissected insects infected with ~200 typing units (TUs), collected in many geographic areas (see Figure S1 in the

supplemental information online) ([1,6] and references within). In 20.2% cases, the location either could not be established or flagellates were found throughout the digestive tract, which could be an artifact of dissection or a mixed infection of trypanosomatids with different tissue specificity. Apart from clearly localized infections in the midgut (50.7%) and hindgut (22.4%), in 5% of cases, the infection was concentrated into a small section at the border between these two parts of the intestine. Infections of Malpighian tubules (~2%) were often combined with parasite occurrence in adjacent part of the midgut and/or hindgut.

predatory or scavenging life styles, which facilitate parasite transmission. The second group, the suborder Nematocera, includes many hematophagous flies that also transmit *Leishmania* and some *Trypanosoma* spp. The most likely evolutionary scenario posits that the transition from a monoxenous to a dixenous life style occurred in female flies able to feed on both vertebrate blood and plant juices. The remaining monoxenous species come from Siphonaptera (fleas), Blattodea (cockroaches), and Hymenoptera (bees), with only a few findings known from Mecoptera (scorpionflies) and Lepidoptera (butterflies) (Figure S2).

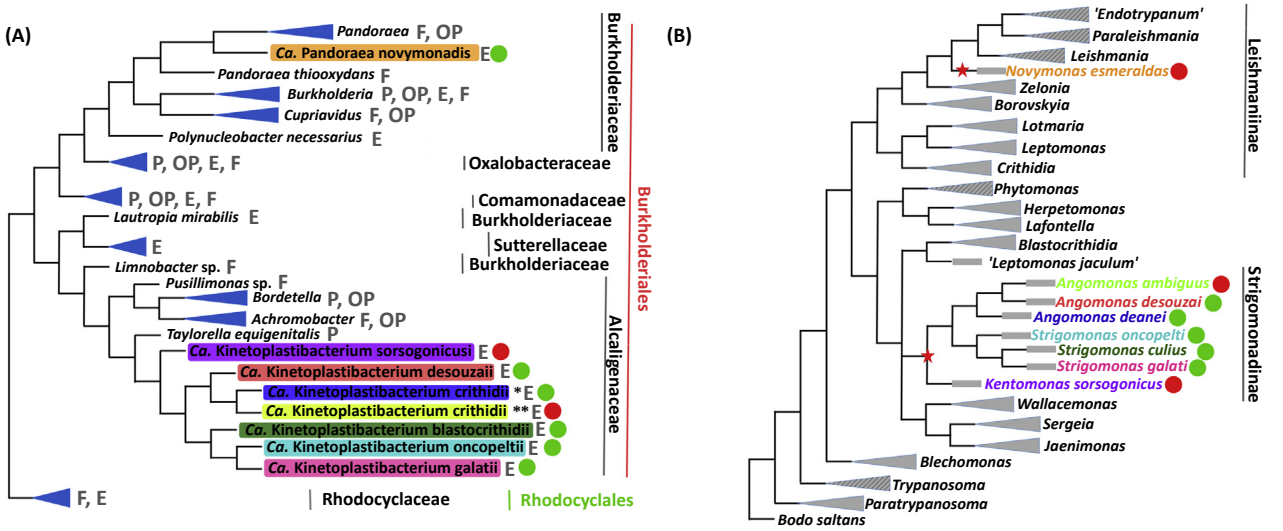
The vast majority of TUs are known from a single host species collected in a single locality. This phenomenon can be interpreted either as evidence for narrow host specificity or, more likely, as a consequence of insufficient sampling. Although there are well documented cases of high host specificity [10–12], a significant fraction of TUs has been found in two or more host species [13], and some geographically widespread TUs even parasitize different families, reflecting a broad spectrum of host–parasite associations (Figure S2).

Molecular phylogenetics of the fast-growing number of TUs guides an ongoing taxonomic overhaul, with more new genera described within the last decade than within the last 100 years [4,5,14–16], providing a clearer view of trypanosomatid evolution and diversity (Figures 1 and 2). Several major clades have been identified, and some of these received formal taxonomic status (*Borovskyia*, *Jaenimonas*, *Kentomonas*, *Lafontella*, *Lotmaria*, *Novymonas*, *Wallacemonas*, and *Zelonia*), whereas in others this step has so far been precluded by the lack of cultures. The genus *Paratrypanosoma* stands out from the rest of the family as the earliest diverging lineage known to date [17].

Bacterial Endosymbionts and Viruses

Until recently, the subfamily Strigomonadinae, subdivided into the genera *Strigomonas*, *Angomonas*, and *Kentomonas*, was thought of as the only trypanosomatid group which hosts the obligatory endosymbiotic β-proteobacteria of the family Alcaligenaceae [18]. However, it has been shown that endosymbiont acquisition occurred at least once more in trypanosomatid evolution, namely in the member of the recently described genus *Novymonas*. This monoxenous trypanosomatid harbors bacteria belonging to the family Burkholderiaceae, only distantly related to the family Alcaligenaceae of the '*Ca.* Kinetoplastibacterium' spp. [16] (Figure 2). That both '*Ca.* Kinetoplastibacterium' spp. and '*Ca.* Pandoraea novymonadis' belong to the same order Burkholderiales is not surprising since bacteria from this group have widely different life styles and occupy diverse ecological niches (Figure 2).

The two endosymbiotic systems differ substantially not only in terms of involved members. While there is tightly regulated division of the bacterium in Strigomonadinae [19], such a fine-tuned, host-mediated control is clearly absent in the *Novymonas* and Pandoraea association, indicating a more recent origin [16]. Instead, *Novymonas* seems to exert only limited control over the cytoplasmic bacteria, primarily via lysosomal degradation. The genomes of both endosymbionts are characterized by a reduced size, low GC content, multiple gene losses, and consequent disruption or loss of some metabolic pathways [20]. The interdependence of both partners is reflected by



Lifestyle:
F - free-living
P - parasitic
OP - opportunistic pathogen
E - endosymbiont

Life cycle:
- Monoxenous (= one-host) genus
- Monoxenous (= one-host) species
- Dixenous (= two-host) genus

Genome sequence:
- Publicly available
- Unavailable

★ - Origin of endosymbiosis
* - *Ca.* K. crithidii of *Angomonas deanei*
** - *Ca.* K. crithidii of *Angomonas ambiguus*

(C)

Feature	*Ca.* Kinetoplastibacterium	*Ca.* Pandoraea novymonadis
General features		
Host	Strigomonadinae	*Novymonas esmeraldas*
Number of symbionts per host cell	1	0–15
Localization within the host cell	Cytoplasm; in the proximity of glycosomes	Vacuoles
Cell wall	Absent	Present
Cell devision	Synchronous with the host	Not coordinated with the host
Mode of transmission	Vertical	Vertical
Genomic features		
Assembly size, Mb	~0.8	1.16
# of protein-coding genes	690–732	968
# of tRNA genes	43–44	43
# of pseudogenes	2–19	13
GC content, %	30–33	44
Metabolic features		
Synthesized amino acids	Gly, His, Lys, Phe, Ser, Trp, Tyr	Arg, Gln, Glu, Gly, His, Ile, Leu, Lys, Phe, Ser, Thr, Trp, Tyr, Val
Peptidoglycan synthesis	DAP type	DAP type
Heme biosynthesis pathway	Partially present	Partially present
Polyamine synthesis	Absent	Absent
Purine synthesis	Present	Present
Vitamin requirements	Thiamine, nicotinic acid, biotin	-
FA β-oxidation	Absent	Absent

(D)

Feature	SHTs	RTs
General features		
Subpellicular microtubular corset	Absent at the site of contact between mitochondrion and plasma membrane	Present except for the area of a flagellar pocket
Paraflagellar rod	Reduced	Absent in amastigote stages of *T. cruzi, Leishmania* spp. and *P. confusum* and reduced or absent in haptomonads of the latter
Mitochondrion	Enlarged and extensively branched; loose kDNA arrangement	A shape of a mitochondrion and kDNA topology differ among species and developmental stages
Genomic features (^)		
Assembly size, Mb	~24 for *Angomonas;* ~26 for *Strigomonas*	~30.6
# of protein-coding genes	~14 486	6 381–11 585
Metabolic features (^)		
Amino acid requirements	Met	Arg, His, Ile, Leu, Phe, Trp, Tyr, Met
Heme biosynthesis pathway	Partially present	Absent; partially (3 genes) present in Leishmaniinae
Vitamin requirements	Thiamine, nicotinic acid, biotin	Riboflavin, pantothenic acid, pyridoxamine, folic acid, thiamine, nicotinic acid, biotin
Requirements for purines	-	Absolute

SHTs - Symbiont-harbouring trypanosomatids
RTs - Regular trypanosomatids
DAP - Diaminopimelic acid
FA - Fatty acid
^ - In case of SHTs data originates from the studies of the Strigomonadinae subfamily since the genome of *Novymonas esmeraldas* is not available

Trends in Parasitology

Figure 2. Endosymbiotic Bacteria. (A) Phylogenetic relationships among the trypanosomatid endosymbionts belonging to the β-proteobacterial order Burkholderiales. The background colors correspond with the coloring scheme in panel (B) and reflect host–endosymbiotic relationships. (B) Phylogenetic relationships of trypanosomatids. Eubodonid *Bodo saltans* served as an outgroup. Monoxenous genera and species are marked with gray triangles and rectangles, respectively, while triangles are shaded for dixenous genera. Two independent origins of endosymbiosis in Trypanosomatidae are marked with red stars. (C) A comparative table for '*Ca.* Kinetoplastibacterium' spp., the endosymbionts of the subfamily Strigomonadinae, and '*Ca.* Pandoraea novymonadis', endosymbiont of *Novymonas esmeraldas* of the subfamily Leishmaniinae. (D) A table summarizing similarities and differences between symbiont-harboring and symbiont-free trypanosomatids.

putative provision of ATP and phosphatidylcholine to the bacterium, which is likely reciprocated by providing the protist with heme, vitamins, purines, and some essential amino acids (Figure 2). The molecular basis of the host–endosymbiont relationships has been studied so far only in the subfamily Strigomonadinae, for which the genomes are available [21–23].

Leishmania RNA viruses such as LRV1/2 were shown to aggravate the symptoms and prognosis in various leishmaniases due to an augmented immune response [24]. It is reasonable to assume that exploring viruses in monoxenous flagellates could shed light on the origin of LRV1/2 and other viruses. A recent broad survey revealed that monoxenous trypanosomatids indeed host Narna-like and Tombus-like viruses, several representatives of the order Bunyavirales, and a unique Ostravirus (Figure 1) [25]. All of these viruses were likely acquired independently and from various sources, including insects, terrestrial invertebrates, and fungi. However, none of them is related to *Leishmaniavirus* (LRVs) [25]. The capacity of *Leptomonas pyrrhocoris* to be simultaneously infected by at least two RNA viruses (Figure 1) testifies to the usefulness of this model for exploring interactions of the viruses with each other and their cosmopolitan hosts [10]. A similarly unexpected finding was the presence of an endogenous element related to the Tombus-like virus 1 in the subtelomeric region of one *L. pyrrhocoris* chromosome [25,26].

Since no viruses related to the medically important LRVs were found outside the *Leishmania* group, the most parsimonious scenario implies that these viruses were acquired after the separation of dixenous leishmanias from their monoxenous relatives. *Leptomonas seymouri* is an emerging opportunistic coinfectant of *Leishmania donovani* in visceral leishmaniasis patients [27]. The only virus recovered from the humans with mixed infection was a Narna-like virus of *L. seymouri* [28]. This observation poses the intriguing question of whether this virus can also play a role in the development of leishmaniasis, and if so, how similar the process is to the well-studied cases of LRV1/2. Since the viral load was extremely high [29], it is likely that this virus could also manipulate the vertebrate immune system in order to give an advantage to the *Leishmania* parasite.

Genomic Diversity

Nowadays, genome sequences represent an indispensable component of the toolbox used for understanding various aspects of trypanosomatid biology. Since the publication of the TriTryp genomes [30], most sequencing efforts were directed primarily towards the medically relevant species [31–33]. Thus, the numbers of available *Leishmania* and *Trypanosoma* genomes are >30 and 20, respectively. However, this bias of human pathogens dominating publicly available genomes has started to change. Four genomes are currently available for the plant parasite *Phytomonas* [34,35], and the genomes of monoxenous *Leptomonas pyrrhocoris*, *L. seymouri*, *Paratrypanosoma confusum*, *Lotmaria passim*, *Crithidia bombi*, and *C. expoeki* have been published very recently [26,29,36–38]. In addition, the genomes of *Crithidia fasciculata*, *Herpetomonas muscarum*, *Blechomonas ayalai*, and several endosymbiont-harboring Strigomonadinae are also available in databases (Figures 1 and 2).

The monoxenous and dixenous Leishmaniinae have an average genome of 31.7 Mb, not deviating considerably from this size (Figure 1). In contrast, the *Trypanosoma* spp. genomes are more variable in size, ranging from ~14 Mb in *T. rangeli* to ~47.5 Mb in *T. vivax*, while *Phytomonas* spp. possess highly streamlined genomes of ~18 Mb [35]. The total number of protein-coding genes is somewhat higher in trypanosomes, with *T. congolense* having the most with 11 585 proteins, while phytomonads carry only about half that number, and *Leishmania* spp. encode 8600 proteins on average. Gene duplications, which may reach

more than 30% in some *Trypanosoma* spp. and ~14% in the *Leptomonas*/*Crithidia* clade, mainly contribute to these differences (Figure 1). Considering the lack of gene regulation at the level of transcription, a changing gene dosage via gene duplications or even ploidy alterations is considered to be the important mechanism of gene expression modulation in trypanosomatids, along with the post-transcriptional control [39,40].

The genomes of monoxenous trypanosomatids proved to be instrumental for our understanding of the emergence and evolution of their dixenous kin. Thus, the genomic data available for the *Leptomonas*/*Crithidia* clade has allowed delineation of novel putative virulence factors in their sister lineage, the genus *Leishmania* [26]. A comparative genomic study with an underlying assumption that at least some of the *Leishmania* virulence factors might have been gained at the *Leishmania* node led to the identification of more than 20 candidate genes, albeit mostly with unknown functions. A gene encoding a putative ATP/GTPase was experimentally proven to affect *L. mexicana* growth *in vitro* and its ability to achieve maximal levels of parasite load *in vivo* in both mice and insects [41].

The orthologous groups (OGs) gain and loss analysis, performed on an expanded dataset containing annotated proteins from 39 trypanosomatids that include 8 monoxenous species, emphasizes the need for follow-up experimental studies aiming at better understanding the functions of trypanosomatid-specific proteins (Figure 1). Using the genomes of the members of *Leptomonas/Crithidia* clade as an outgroup, we have delineated 100 OGs gained at the *Leishmania* node. Out of those, 96 are hypothetical proteins, reflecting once again the scarcity of functional data for many trypanosomatid protein families. Of note, the A2 protein, gained at the *Leishmania* node (Figure 1 and Table S2), has been shown to affect the ability of *L. donovani* to establish visceral infection, and the corresponding gene is pseudogenized in *L. major* [42]. A possible involvement of other genes gained at this node in *Leishmania* virulence remains to be investigated. It is worth mentioning that four OGs gained at the *Leptomonas*/*Crithidia* node include putative RNA-binding proteins (RBPs), which are known as regulators of the *T. brucei* life cycle [43]. Genome-wide screening for the CCCH-type zinc finger RBPs in the TriTryp genomes revealed that most were already present in the ancestor of these trypanosomatids. However, the lineage-specific repertoire of RBPs is shaped by gains, duplications, and losses [44]. Apparently, this is also valid for monoxenous trypanosomatids, possibly reflecting the peculiarities of their insect-confined life cycles. We have also found an OG containing a putative *Crithidia*/*Leptomonas*-specific amastin surface glycoprotein exhibiting 65% similarity to the previously identified *C. fasciculata*-restricted *ama17* [45]. This finding provides support for the idea that, although the amastin repertoire is elaborated in the genus *Leishmania* and these proteins are thought to be crucial for the survival inside the vertebrate cells, they might also play a (more ancestral) role in the interaction with the insect hosts. We explored a similar situation with receptor adenylate cyclases (Box 2).

Despite the multiple gene gains and losses, the observed overall synteny levels are high both within and between monoxenous and dixenous species [26,29,38]. This conservation of genome structure may reflect the organization of genes into polycistronic clusters and nearly complete absence of *cis*-spliced introns (with the notable exception of poly(A) polymerase and DEAD/H RNA helicase).

Genomic data provide important insight into the structural and physiological peculiarities of monoxenous trypanosomatids. In comparative studies based on the whole-genome data from various trypanosomatid species and isolates of different geographic origin, genes associated with the flagellum and cytoskeleton were shown to be under positive selection pressure [26,38].

Box 2. A Case Study: Receptor Adenylate Cyclases

Trypanosomatid receptor adenylate cyclases (RACs) are predicted to be key players at the parasite–host interface [68]. They bear a conserved structure formed by a transmembrane domain for integration into the plasma membrane, a cytosolic catalytic (AC) domain for ATP to cAMP conversion, and a ligand-binding extracellular domain (Figure I). Upon unidentified ligand binding, cAMP concentration increases within microdomains radiating from the AC domain to stimulate unknown signaling pathways. Phosphodiesterases hydrolyse cAMP to AMP, preventing the secondary messenger's diffusion to maintain microdomains [69]. RACs and other cAMP metabolizing/interacting proteins are well studied in *Trypanosoma brucei*, prompted by an RAC being encoded by expression site-associated gene 4 (ESAG4), which controls proliferative slender-bloodstream stage cytokinesis [70] and belongs to 11 ESAGs forming a highly-expressed subtelomeric gene array with a variant surface glycoprotein gene [71]. cAMP hydrolysis products participate in quorum sensing, prompting differentiation from slender into the tsetse-fly-infectious stumpy form [72,73]. African trypanosomes encode 34–96 RACs, including ESAG4 in *T. brucei* (Figure 1), a large quantity suggested to be spurred by pressure to survive multifarious threats from mammalian hosts [71]. RACs are differentially expressed in early and late procyclic *T. brucei* that infest different parts of the alimentary tract [74]. Some RACs are negative regulators of social motility, a collective movement in restrictive semisolids only observed in procyclics [69,74]. However, whether this behavior is an adaptation for navigation within the insect *in vivo* remains unclear. Nevertheless, RACs are likely important at the parasite–insect host interface, underscored by their restricted expression in sandfly-dwelling *Leishmania donovani* promastigotes [75]. To investigate this idea, we searched for ESAG4 homologs in trypanosomatid genomes (Figure 1). Triatomine-bug-transmitted *Trypanosoma rangeli* and *T. cruzi* have 9–12 RACs. The 1 or 2 *Phytomonas* RACs may facilitate parasite–insect interaction as their plant hosts lack cell-mediated immunity. Within Leishmaniinae, *Leishmania* spp. bear 5–8 RACs, paralleling the range in *Crithidia* (1–10) and *Leptomonas* spp. (3–9). Two RACs found in flea-infecting *Blechomonas ayalai* further support their role at the trypanosomatid–insect interface. Intriguingly, *Paratrypanosoma confusum* lacks any RAC, contradicting this hypothesis. Thus, monoxenous trypanosomatids provide a valuable perspective in understanding RACs.

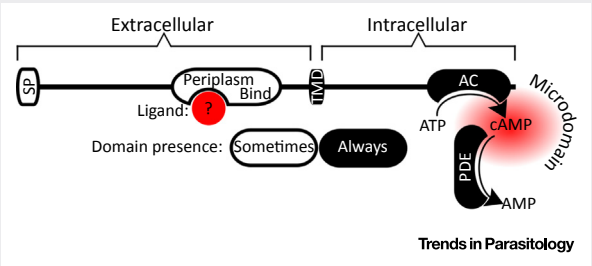


Figure I. Scheme of a Receptor Adenylate Cyclase with Common Motifs. Abbreviations: SP, signal peptide; AC, adenylate cyclase catalytic domain; TMD, transmembrane domain; PDE, phosphodiesterase. All receptor adenylate cyclases contain TMD and AC, while SP and periplasm-binding protein-like domain are present only in some.

This finding seems to be counterintuitive considering a widely known conservation of these structures. However, in trypanosomatids, the flagellum participates not only in cell motility and cell division, but also plays an important role in host–pathogen interactions [46,47]. Moreover, the flagellum and flagellum-associated cytoskeleton undergo an extensive remodeling depending on the cultivation conditions [36]. Indeed, the limited data available from monoxenous species indicate that their flagellum is more flexible than what would expected by extrapolation of knowledge from their well-known dixenous relatives, being able to form complex attachment plaques [48] and alter their length [29] (Figure 3).

The few monoxenous genomes have yielded other surprises. A prominent example is that of *Blastocrithidia* sp., which reassigned all three stop codons into sense codons. The UAG and UAA codons were predicted to code for glutamate, and the UGA codes for tryptophan. Strangely enough, UAA is still used as a stop codon [49]. This is the first case of such a phenomenon outside of ciliates, which are notorious for their codon reassignments [50]. However, unlike ciliates, *Blastocrithidia* sp. can be easily cultivated, has a standard genome and transcriptome, and is likely amenable to genetic manipulations as are other monoxenous trypanosomatids. These qualities provide clear advantages to make *Blastocrithidia* spp. promising models for addressing the fundamental questions about codon reassignment, such

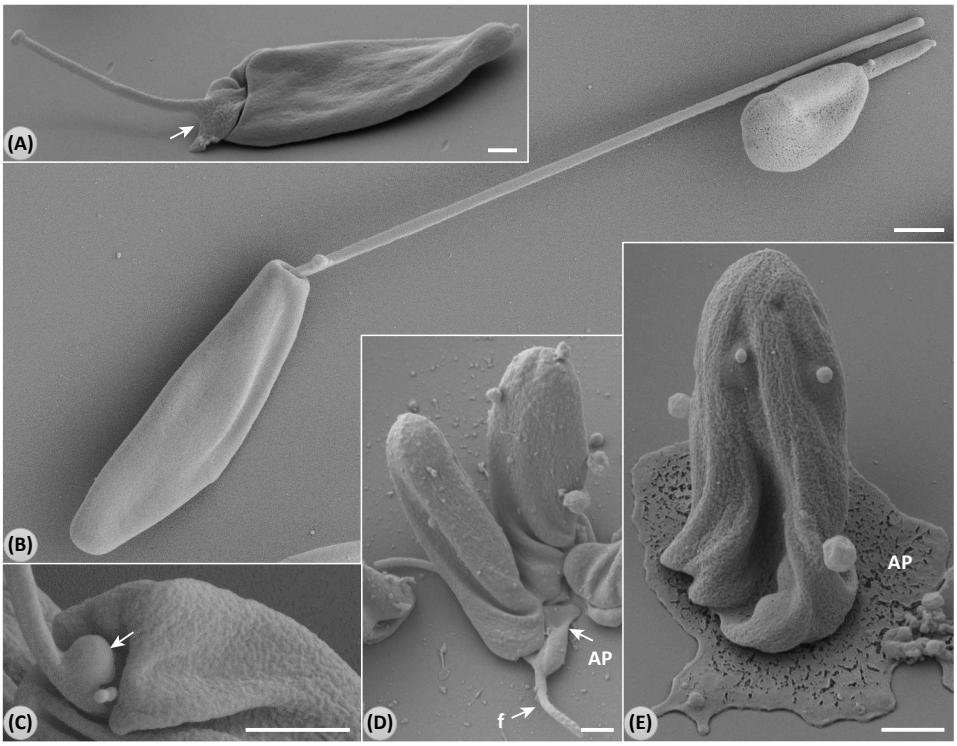


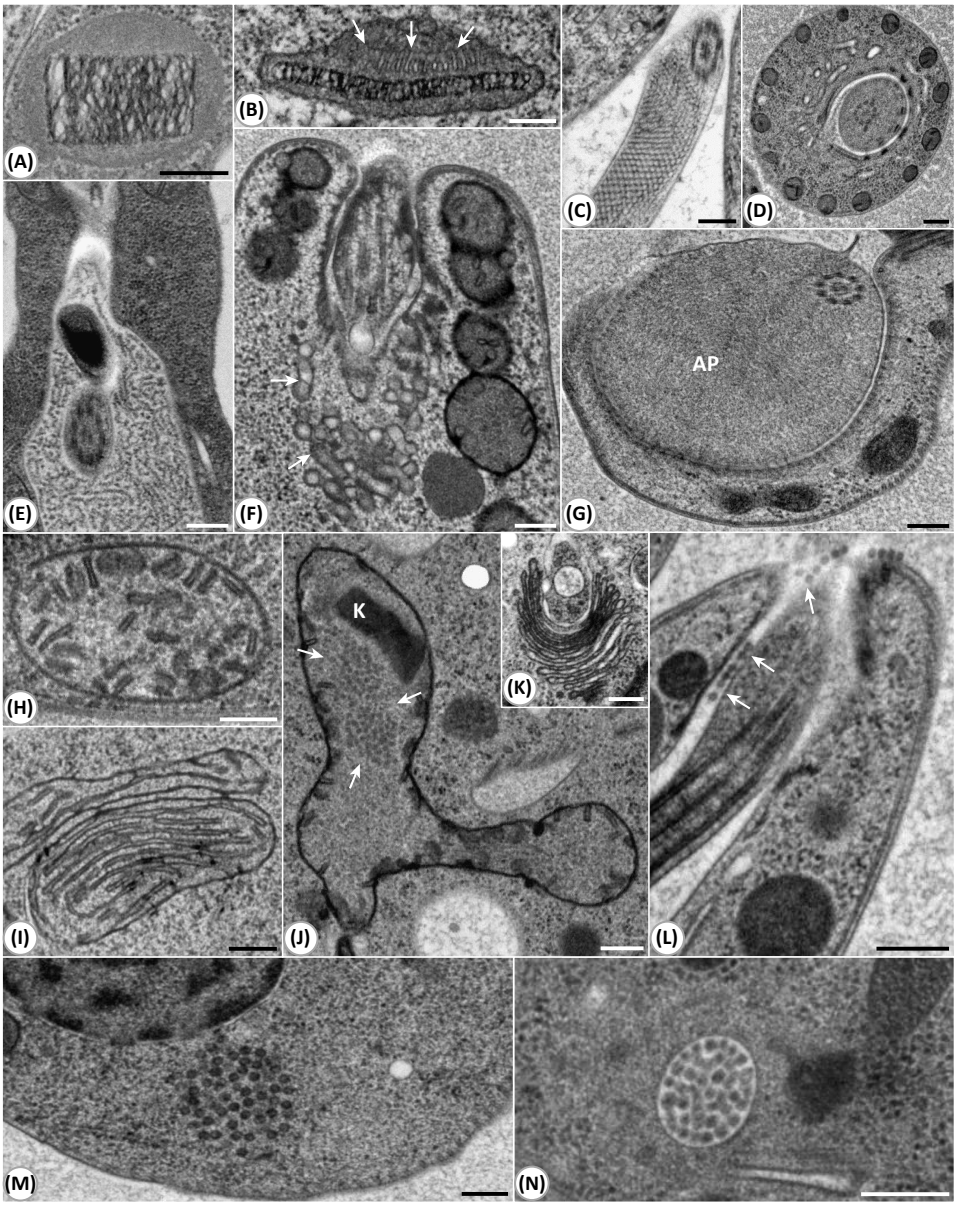
Figure 3. Diverse Morphology (I). (A) The promastigote of *Paratrypanosoma confusum* (strain CUL-13) with a prominent bulge (arrow) at the base of the flagellum, adorned with a short ridge. (B) Two promastigotes of *Wallacemonas raviniae* (strain ECU-07) with different cell shapes and flagellar lengths. (C) A round bulge at the base of the flagellum (arrow) of an unnamed trypanosomatid CB-05. (D) Two haptomonads of *Paratrypanosoma confusum* in the process of attachment to the surface. Note the transformation of the flagellar bulge into the attachment pad (AP) and the remaining part of the flagellum (f). (E) Characteristic upright position of the *P. confusum* haptomonad with an extensive attachment pad (AP). All scanning electron microscopy pictures; scale bar is 1 μm.

as the exact mechanism of translation termination, the distribution and frequency of in-frame stop codons, and the mechanisms by which the ribosome can distinguish between in-frame and termination stop codons.

The optimistic outlook for establishing *Blastocrithidia* sp. as a *bona fide* model is that transgenic cell lines have been already generated in two monoxenous trypanosomatids: *C. fasciculata* [51] and *L. seymouri* [29]. Furthermore, we predict that a tetracycline-inducible system for controlling transgene expression, which is routine for *T. brucei* [52], will be possible to establish in these insect-infecting trypanosomatids as has been done for *Leishmania* [53,54].

Ultrastructural and Biochemical Diversity

Morphological data, now available for a growing number of monoxenous trypanosomatids, may educate functional studies, for example, by identifying species in which a given cellular feature is present in an unusual form. Yet, the features, although variable, do not show a drastic departure from a common theme. Thus, the ultrastructure of the kinetoplast (k) DNA does vary substantially, reflecting the species-specific size of the minicircles, which, nonetheless, are invariably concatenated, and the kDNA is represented *in vivo* by a conserved disk-shaped structure (Figure 4A,B). A similar situation applies to the paraflagellar rod, the size and structure



Trends in Parasitology

Figure 4. Diverse Morphology (II). (A) Thick kDNA disk of *Crithidia otongatchiensis* (strain ECU-08) composed of large minicircles. (B) Thin kDNA disk of an unnamed trypanosomatid SB-10 composed of small minicircles. Note the arrangement of mitochondrial cristae in one face of the kDNA disk (arrows). (C) Prominent paraflagellar rod of *Jaenimonas drosophilae* (strain dfal-01) with a lattice-type structure. (D) Regularly spaced and peripherally distributed single mitochondrial network of *Kentomonas sorsogoni* (strain MF-08). (E) Deep flagellar pocket of *Wallacemonas raviniae* (ECU-07) filled with granular material of unknown composition. (F) Shallow flagellar pocket of *Crithidia otongatchiensis* surrounded by numerous tubular-shaped networks of endocytic vesicles (arrows). Note numerous cross-sections of the mitochondrial network with short peripheral cristae. (G) Flagellum of the haptomonad stage of *Paratrypanosoma confusum* (CUL-13) extended into an adhesive pad (AP). (H) Cross-sectioned mitochondrial network of *C. otongatchiensis* packed with short disk-like cristae. (I) Longitudinal section of mitochondrial network of an unnamed trypanosomatid containing numerous very long cristae. (J) Section through the mitochondrion of *Crithidia pragensis* (strain MCZ-11) with a cluster of virus-like particles (arrows) in the lumen adjacent to the kDNA disk (K). Note the paucity of short peripheral cristae. (K) Single large

(See figure legend on the bottom of the next page.)

of which vary from a prominent character (Figure 4C) to a repressed form [55]. Invariably there is a single reticulated mitochondrion per cell, yet substantial interspecific differences exist in the number of cristae (Figure 4H–I). Species in which a given cellular structure is specifically modified, enlarged, or otherwise altered, are worth particular attention. The flagellar pocket of *Crithidia otongatchiensis* is surrounded by a uniquely expanded network of endocytic vesicles (Figure 4F), while the same structure in *Wallacemonas raviniae* is packed with granular material (Figure 4E). Particularly interesting is *P. confusum*, with an unusually large Golgi apparatus (Figure 4K). This basal-branching trypanosomatid has a capacity to massively restructure its flagellum from a flagellar bulge (Figures 3A and 4G), which, during the life cycle, (reversibly) transforms into an extensive sticky adhesive pad (Figure 3D,E) and contains other putatively ancestral characters [36].

Morphologically, the endosymbiont-bearing trypanosomatids of the subfamily Strigomonadinae (Figure 2) clearly stand apart from the rest. They have a loosely organized kDNA disk [56], a repressed paraflagellar rod [55], and a mitochondrion that disrupts its subpellicular microtubule corset (Figure 4D) [18]. It is likely that this unique morphology reflects the presence of its endosymbiont, or may have been a primitive trait that allowed invasion by the bacterium.

Ultrastructural analysis is also informative regarding viruses that are unexpectedly frequent in monoxenous species (Figure 1) [25]. Clusters of virus-like particles, either located in membranous vesicles (Figure 4N) or freely in the cytoplasm (Figure 4M) have been observed in *Leptomonas moramango* and *P. confusum*, respectively. We were able to capture the release of virus-like particles into the flagellar pocket (Figure 4L), which is not surprising given the frequently periflagellar position of the virus-containing vesicles. Rarely, viruses seem to reside in the mitochondrial lumen, such as in *Crithidia pragensis* (Figure 4J).

In general, most features well known from trypanosomes and leishmanias are also present in monoxenous trypanosomatids and, while exhibiting variability, no truly novel structures have been found so far. The general flexibility of their metabolism is reflected in the trend of species with a high rate of glycolysis having a less active respiratory chain, or that a key enzyme, trypanosome alternative oxidase, was retained only by some monoxenous trypanosomatids. Moreover, this group of flagellates seems to be multipotent in terms of mitochondrial functions, their potential being influenced by different nutrients, hosts, and growth temperatures [57,58].

Concluding Remarks

Insect trypanosomatids are a diverse group of widely distributed protists preferentially found in hosts with life styles that facilitate transmission. Although Heteroptera and Diptera remain the most frequent hosts, numerous other groups also harbor these flagellates. The host–parasite interactions are also diverse and, accordingly, the parasite's impact on the insect can be varied. Moreover, the host specificity can also vary, and nonspecific associations are not uncommon. Monoxenous species are subdivided into several major clades forming new subfamilies in the phylogeny-based classification system under development, which gradually replaces the traditional morphotype-based system. A new host–endosymbiont association discovered recently is expected to shed new light on the origin and advantages of such relationships. A number of outstanding questions remain (see Outstanding Questions). The true extent of

Outstanding Questions

What is the origin of *Trypanosoma*, and when and how did the dixenous lifestyle evolve within Trypanosomatidae?

What are the true diversity and ecological impact of monoxenous trypanosomatids?

What is the rationale for repurposing of stop codons into sense codons in *Blastocrithidia*, and how does translation terminate?

What is the origin of LRV1/2 viruses in *Leishmania* species, and what are the functional roles of viruses in various other trypanosomatids?

What are the underlying mechanisms of genome reduction in endosymbiotic bacteria of trypanosomatids?

Golgi apparatus of *P. confusum*. (L) Flagellar pocket tip of *P. confusum* with virus-like particles (arrows). (M) A cluster of virus-like particles located within the perinuclear cytoplasm of *Leptomonas moramango* (strain MMO-09), not enclosed by a membrane. (N) A membrane-bound vesicle in the cytoplasm of *P. confusum* filled with virus-like particles. All transmission electron microscopy pictures; scale bar is 250 nm.

trypanosomatid diversity is still unknown, and intensification of efforts in this direction is needed, with the surveys expanding to new geographic regions and hosts groups. The impact of insect parasites on ecosystems is not well understood, although there is progress in this direction. Research in these areas would greatly benefit from the development of a high-throughput genotyping protocol for analysis of environmental samples that would replace the current, rather slow and tedious procedure. Another important group of questions pertain to the origin and evolution of parasitism. Although comparative genomics has brought new insights into the origin of dixenous parasitism in *Leishmania*, the origin of *Trypanosoma* remains as obscure as ever. A solution to this problem lies in the search and analysis of additional basal trypanosomatid lineages (such as *Paratrypanosoma*) and in construction of a robust phylogenetic (or phylogenomic) framework for the family. The latter would also call for expanding the range of the sequenced genomes to include all major trypanosomatid clades (currently known and likely discovered in the future), along with the deep-branching lineages and the appropriate outgroup bodonids.

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