Review

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Diversity and phylogeny of insect trypanosomatids: all that is hidden shall be revealed

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Monoxenous trypanosomatids, which are usually regarded as benign dwellers of the insect alimentary tract, represent a relatively obscure group within the family Trypanosomatidae. This field of study has long been in disarray with the genus level taxonomy of this group remaining artificial, species criteria elusive, host specificity and occurrence poorly known, and their diversity mostly unexplored. The time has arrived to remedy this situation: a phylogenetic approach has been applied to taxa recognition and description, and a culture-independent (PCR-based) approach for detection and identification of organisms in nature has made it feasible to study the diversity of the group. Although more than 100 typing units have been discovered recently, these appear to represent a small segment of trypanosomatid biodiversity, which still remains to be uncovered.

Trypanosomatids – a distinct group of protists

The history of research on insect trypanosomatid biodiversity has quietly passed its sesquicentennial milestone a few years ago. Findings of trypanosomatids in insects were initially documented in 1851 by Burnett [1], and the first genera (*Leptomonas* and *Herpetomonas*) were established in 1880 by Kent [2], although not exactly with the same meaning that these names imply today. A burst of publications presenting new species of insect trypanosomatids and further development of the taxonomic system occurred in the first half of the 20th century. At that time, and during the next few decades, the main method of investigation was light microscopy. This period culminated in the mid-1960s with the establishment of the morphotype-based taxonomic system of Trypanosomatidae (Box 1, Figure I) by Hoare and Wallace [3,4] who redefined the genera on the basis of specific morphotypes and life cycles. The genera *Leishmania* and *Trypanosoma* represent dixenous parasites of vertebrates (see Glossary) including important human pathogens. Dixenous parasites of plants are assigned to the genus *Phytomonas*. Monoxenous parasites are divided among the genera *Blastocrithidia*, *Crithidia*, *Herpetomonas*

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Glossary

Apomorphy: a derived characteristic of a taxon; for example, any feature novel to a taxon.
Bootstrap analysis: in phylogenetic reconstruction, this is a procedure to evaluate the level of support provided by the data for a particular element of the tree topology. It involves omission of a part of the data with replacements drawn from the remaining data. Frequent appearance of a clade ('bootstrap support') in a majority consensus tree is often (over)interpreted as a confidence level in the tree topology.
'Camera lucida': an optical device attached to the eye piece of a microscope. It operates by superimposing an image of the subject being viewed upon a drawing surface. Such drawings were often used in the past to record the morphology of protists in lieu of microphotography.
Choanomastigote: a morphotype characterized by barley-shape cells with a wide flagellar pocket and kDNA pre-nuclear or adjacent to the nucleus.
Clade: a group consisting of an ancestor and all its descendants. A clade is monophyletic by definition.
Coprophagy: the consumption of feces.
Dixenous parasite: a parasite with a life cycle split between two host species; for example, as during insect-mediated transmission among vertebrates.
Endomastigote: a morphotype characterized by round to oval cells with a very short flagellum convoluted around the nucleus and not extending outside of a flagellar pocket.
Epimastigote: a morphotype characterized by prolonged cells with the lateral opening of a flagellar pocket, an apposed flagellum, and pre-nuclear kDNA. A short undulating membrane can be present.
Genus: a low-level taxonomic rank used in the biological classification; genus lies above species and below family.
Monophyletic, monophyly: a monophyletic group includes an ancestor and all its descendants, or any two or more groups that share a common ancestor. Members of monophyletic groups are typically characterized by shared derived characteristics (synapomorphies).
Monoxenous: a parasite that is restricted a single host (invertebrate or vertebrate) during its life cycle.
Necrophagy: the consumption of dead and decaying organic matter.
Opisthomastigote: a morphotype characterized by cells with long and narrow flagellar pocket extending from a post-nuclear (posterior) kinetoplast to the anterior end of the cell.
Opisthomorph: a morphotype similar to choanomatigotes but with posterior kinetoplast.
Paraphyletic, paraphyly: a paraphyletic group consists of an ancestor and the majority of its descendants. In other words, this is a monophyletic group from which one or more of its members are excluded to form separate groups (e.g., due to not sharing a particular derived trait that is present in the remaining members of the paraphyletic group).
Polyphyletic, polyphyly: a polyphyletic group is characterized by one or more character states which represent convergence or reversal traits which appear to be similar but which were not inherited from the last common ancestor. It is neither monophyletic nor paraphyletic.
Post-nuclear (kinetoplast, kDNA): positioning of a kinetoplast between the nucleus and the posterior end – (the end opposite to the protruding flagellum.
Pre-nuclear (kinetoplast, kDNA): positioning of a kinetoplast between the nucleus and the anterior end of the parasite cell (the end with the protruding flagellum).

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Spliced leader RNA (SL RNA): a class of short transcripts (approximately 100 nt in length) which participate in mRNA maturation in kinetoplastids. An SL RNA molecule includes a 39 nt conserved mini-exon which is transferred onto the 5′ end of each mRNA by *trans*-splicing. SL RNA genes are arranged as clusters of multiple tandem repeats (0.2–1.0 kb in length). The intergenic regions of the repeats are nearly identical within the same species but are highly variable between species.
Subfamily: a taxonomic rank below the family and above the genus levels.
Synapomorphy: a derived trait (apomorphy) that is shared by two or more taxa and their most recent common ancestor.
Tribe: a group of closely related genera ranking below the subfamily level.
Trypomastigote: a morphotype characterized by a prolonged cell shape with apposed flagellum emerging from a lateral opening of the posterior flagellar pocket and the post-nuclear kDNA.

and *Leptomonas* (not considering a few small poorly known or dubious genera). The catalog of trypanosomatid species published in 1990 listed 350 named species of insect trypanosomatids [5] with the current number approaching 400. Old species descriptions mainly included only cell dimensions illustrated with 'camera lucida' drawings and, in some cases, observations on the natural history of the parasite, including characterizations of life-cycle stages or some aspects of interaction with the host (a few examples are given in [6–8]). Subsequently, species descriptions began to include ultrastructural details and occasionally some biochemical data (e.g., [9,10]).

Ultrastructure (Figure 1) proved to be important for realization that endoparasitic trypanosomatids, cells with a single flagellum, are related to bodonids, a rather diverse group of free-living and ecto- and endoparasitic protists with two flagellae. The unifying taxon, Kinetoplastea, is mainly defined by the presence of the kinetoplast, a region encompassing the single mitochondrion of a cell and which typically contains a large amount of DNA [11–13]. Ultrastructural synapomorphies, primarily the structure of the paraxonemal rod, also served to assign kinetoplastids to the higher-level taxon Euglenozoa [14]. No specific taxonomic rank is assigned to Kinetoplastea or Euglenozoa in the new rankless classification system [15], which is focused on the higher-order phylogenetic relationships.

Factors defining the diversity of insect trypanosomatids

Two major factors have been implicated in defining biodiversity: the number of available niches and the time allowed for diversification. Because insects, the most numerous class of invertebrates, serve as ecological 'niches' for trypanosomatids, it can be anticipated that the biodiversity hotspots, such as those in the tropics, would in turn promote the highest diversity of the parasites. This is obviously the case; however, the actual extent to which the diversity of the host defines the diversity of parasites remains to be determined. If each insect species hosted at least a single trypanosomatid species, the diversity of the latter would be staggering, potentially reaching millions of species [16]. However, the underlying assumption is not necessarily correct: not all types of insect hosts may be suitable for colonization, and those that are suitable may be infected, at least hypothetically, by only several parasite species of low host specificity. The questions of host specificity of the parasites, and their actual distribution among host taxa, are fundamental but have not been investigated sufficiently. An additional complication has been the lack of clear and objective criteria to distinguish trypanosomatid species.

For a long time, the 'one host – one parasite' paradigm served as a main criterion for recognition of new trypanosomatid species [4,5]. Built into this view was the assumption of a strict host specificity of parasites. In this regard the past descriptions were fully consistent with the 'quality standards' accepted in parasitology at that time. By the turn of the century a few hundred presumptive species were cumulatively reported from just 2500 investigated host species [17], seemingly supporting the notion of an immense diversity. However, it had also been noted that the host distribution is not uniform, and two insect orders (Hemiptera and Diptera) accounted for over 80% of the documented cases [4]. To what extent this reflects the actual occurrence, as opposed to the biased attention of researchers, remains unclear, but there are several factors that would favor these particular host groups.

Of key importance are the aspects of a natural history of potential hosts facilitating or impeding transmission of parasites, which is believed to occur exclusively by ingestion. Viewed from this angle, transmission in dipterans and hemipterans is favored by their habit of feeding on rich organic sources that are often contaminated by excretions from infected hosts. Frequent insect aggregation on their food source (e.g., among dipterans), or their existence in dense localized populations (e.g., among hemipterans), would also facilitate contaminative transmission. Additional infection routes which include coprophagy and necrophagy, as well as predation, are also common in these groups. Once a host is infected, the nutrient-rich content of its intestinal tract would serve to support the effective propagation of parasites. Such favorable conditions are not universal among insects, some of which have solitary life cycles (meeting their conspecifics only for mating), feed on hard-to-digest substrates (such as cellulose), or do not feed as adults. Therefore, a uniform occurrence of trypanosomatids in invertebrate hosts is unlikely, and some groups would be colonized rarely if at all. However, this problem still requires systematic investigation.

Host specificity of insect trypanosomatids

The next question refers to host specificity, defined as capability of a parasite to infect a single or a few closely related host species (narrow specificity) or a variety of hosts (broad specificity). Although temporary survival or passive transmission of monoxenous parasites appear to take place in some cases [18], the high prevalence of these parasites in some insect populations can only be possible due to the ability of trypanosomatids to cause stable infections of individual hosts. In dixenous *Leishmania*, highly specific interactions between the surface glycoconjugates and the host galectin receptors on the surface of microvilli define vector competence and are vital for the successful development of the parasite [19–21]. It is highly likely that the type and the molecular mechanism of such interactions are primarily responsible for host specificity of insect parasites as well. Within a host, insect trypanosomatids are predominantly found in the midgut and hindgut [4,22–24], where they can often be seen as a dense layer of cells lining the intestinal walls with attachment mediated by the flagellae of the parasites. The details of these interactions vary between species. Thus, in

Box 1. The morphology-based taxonomy of Trypanosomatidae

The current 'classical' taxonomic system is based on combination of morphotypes and life cycles: monoxenous, which include a single invertebrate host, and dixenous, which represent a succession of two hosts – invertebrate (serving as a vector) and vertebrate (or plant) [3,78,79]. The morphotypes are characterized by the relative position of the kinetoplast and flagellum, and the cell shape (Figure I). Most genera show a subset of several morphotypes, but only one of these was regarded as typical, serving as a main taxonomic criterion for genus definition [3,11,80]. Eight morphotypes are usually distinguished. Amastigotes, cells with a round body and no emergent flagellum (Figure I, A), represent a typical stage of the genus *Leishmania* Ross 1903 in cells of vertebrate hosts, but were also observed in *Phytomonas* Donovan 1909, parasitizing plants, and in some trypanosomes (e.g., *Trypanosoma cruzi*). In addition to these dixenous genera, amastigotes are frequently found as pseudocysts, in some cases attached to the flagellum of a mother cell (so-called straphanger cysts), in the monoxenous genera *Leptomonas* Kent 1880 and *Blastocrithidia* Laird 1959 (Figure 1, S). Promastigotes, prolonged cells with a narrow flagellar pocket and pre-nuclear kinetoplast DNA (kDNA) (Figure I, P) were regarded as typical for *Leptomonas*, *Phytomonas*, and *Leishmania*, which are differentiated by their life cycles. However, promastigotes are also observed in the monoxenous genera *Crithidia* Legér 1902, *Herpetomonas* Kent 1880, *Wallaceina* Podlipaev, Frolov & Kolesnikov 1990, and *Sergeia* Svobodová, Lukeš & Votýpka 2007, as well as in some dixenous *Trypanosoma* Gruby 1843. Choanomastigotes, barley-shaped cells with a wide flagellar pocket and kDNA pre-nuclear or adjacent to the nucleus (Figure I, C), were considered typical for *Crithidia*. The genus *Wallaceina* was characterized with endomastigotes, round to oval cells with a very short flagellum convoluted around the nucleus (Figure I, EN). Opisthomastigotes, cells with a long and narrow flagellar pocket and post-nuclear kDNA (Figure I, O), served to define *Herpetomonas*. The related opisthomorphs (Figure I, OM) were recognized more recently in the monoxenous genera *Angomonas* Souza and Corte-Real 1991 and in the re-established monoxenous genus *Strigomonas* Lwoff and Lwoff 1931. Epimastigotes, prolonged cells with apposed flagellum and pre-nuclear kDNA (Figure I, E), were thought of as being specific for *Blastocrithidia* and *Trypanosoma*. Finally, trypomastigotes, prolonged cells with apposed flagellum and post-nuclear kDNA (Figure I, T), defined the genus *Trypanosoma*. The latter morphotype, in combination with other criteria, was also used to define two genera which are of a dubious nature: trypomastigotes lacking a pronounced undulating membrane were thought to be characteristic of the monoxenous genus *Rhynchoidomonas* Patton 1910, and intracellular trypomastigotes were thought to be characteristic the dixenous genus *Endotrypanum* Mesnil and Brimont 1908. In each case the concern regarding these genera is the lack of high-quality images (the original drawings notwithstanding) to confirm the validity of these findings. There are no molecular data on *Rhynchoidomonas* that would confirm or negate its separate status, whereas analyses of several genes from cultured strains presumptively representing *Endotrypanum* (*E. schaudinni* and *E. monterogeii*) showed them to be members of the *Leishmania* clade [37,39,68,81].

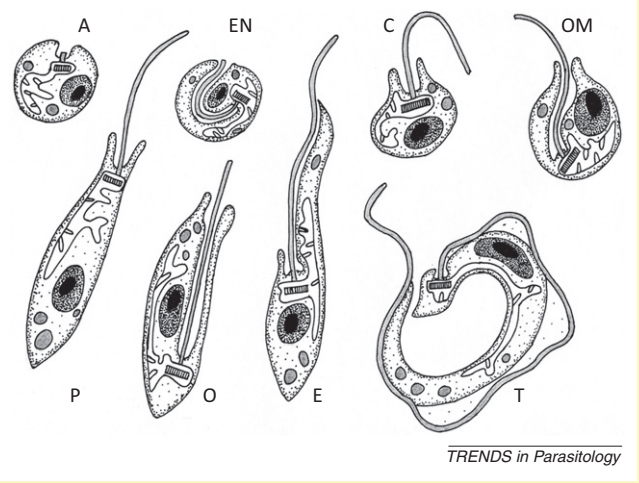

Figure I. Basic morphotypes of trypanosomatids that serve as genus-defining characteristics in the current taxonomy. Abbreviations: A, amastigote; C, choanomastigote; E, epimastigote; EN, endomastigote; O, opisthomastigotes; OM, opisthomorph; P, promastigote; T, trypomastigote.

Blastocrithidia triatomae infecting a reduviid *Triatoma infestans*, there is direct contact between the parasite flagellum and cell body and the host intestinal epithelium [25]. In *Leptomonas wallacei* infections of a lygaeid *Oncopeltus fasciatus*, cell adhesion involves interactions of the flagellum with secreted perimicrovillar membranes of the host, instead of with the microvilli of the epithelium [26]. At the opposite end of the interaction spectrum would be the cases of survival (or even propagation) of trypanosomatids in the intestinal lumen. Such cases, which probably occur among insectivorous reduviids infected by their prey (they host a wider spectrum of trypanosomatids than their herbivorous kin), would represent transient infections. Although the current picture is still rather fragmented and is not yet supported by solid experimental evidence, the emerging view is that host specificity, although being variable, does not depart too far from the venerable one host – one parasite hypothesis. A parasite needs to evolve a molecular fit to its host to establish a stable infection. The surface glycoconjugates of the parasite allow for a specific interaction only with a limited number of hosts with matching receptors. This notion is illustrated by recent data, which show occurrence of *Leptomonas pyrrhocoris* exclusively in the members of the host family Pyrrhocoridae [24,27]. However, such a molecular fit may arise independently in different parasites, as shown by observations of trypanosomatids other than *L. pyrrhocoris* in the Pyrrhocoridae and in a number of analogous cases [24,27,28]. The biodiversity-relevant conclusion is that there seems to be more parasites than suitable hosts, and the overall diversity of parasites is limited by the number of such hosts.

Several reports indicate that some, presumably monoxenous, species are capable of at least limited propagation in vertebrates (Table S1 in the supplementary material online). Additional analyses are required to shed light on this issue.

Phylogenetic solution to taxonomic problems

In trypanosomatid systematics, as well as in biodiversity studies, a serious issue that plagued the host- and morphology-based approach was the dearth of informative characters to characterize the taxa (species and genera) and the lack of objective criteria to draw lines between the taxa [29]. Only a few basic shapes or 'morphotypes' were recognized, and these were used as one of the two major characters for genus designation, the second being the host type (Box 1, Figure I). The caveat of a system based on traits with limited variability is that it forces all trypanosomatids into only a few genera, potentially ignoring

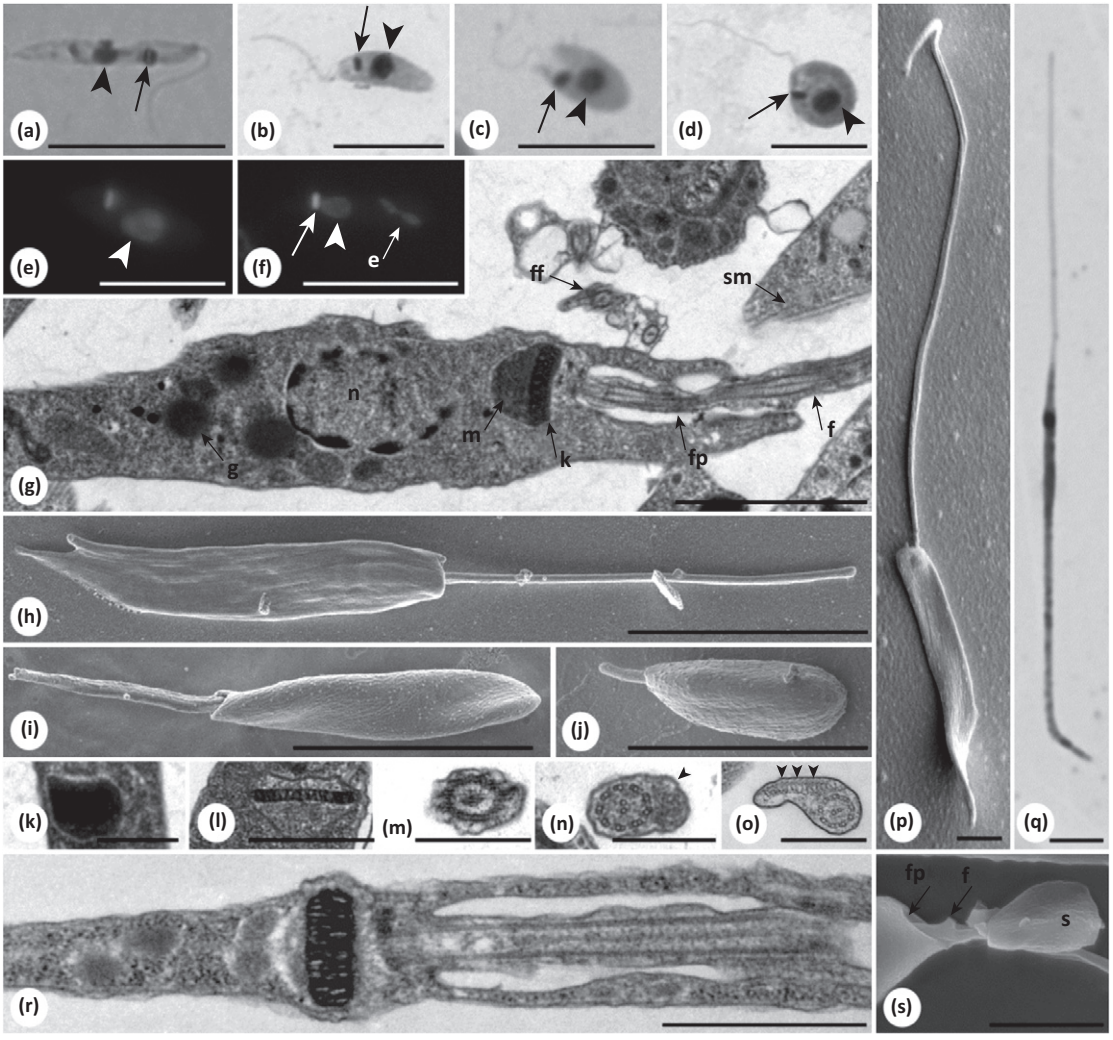

Figure 1. Morphology and ultrastructure of trypanosomatids isolated from true bugs, mosquitoes, and fleas. Flagellates were visualized by staining with Giemsa (a–d,q) or 4′,6-diamidino-2-phenylindole (DAPI) (e,f) (arrowhead and arrow point to nucleus and kDNA, respectively), and by transmission (g,k–o,r) and scanning (h–j,p,s) electron microscopy. **(a)** Elongated promastigote of *Leptomonas costaricensis* from *Ricolla simillima* (Heteroptera: Reduviidae). **(b)** Promastigote of *Leptomonas* cf. *lactosovorans* from *Pachygrontha barberi* (Heteroptera: Lygaeidae). **(c)** Oval choanomastigote of *Crithidia abscondita* from *Largus* sp. (Heteroptera: Largidae). **(d)** A round cell of *Crithidia permixta* from an unidentified host species (Heteroptera: Miridae). **(e)** *Leptomonas jaderae* from *Jadera obscura* (Heteroptera: Rhopalidae). **(f)** *Blastocrithidia culicis* from *Aedes vexans* (Diptera: Culicidae), e – bacterial endosymbionts (two are discernible). **(g)** Longitudinal section through *Leptomonas acus* from an unidentified host species (Heteroptera: Miridae) showing typical ultrastructural characters. **(h)** *Leptomonas bifurcata* from *Pachypoda* sp. (Heteroptera: Miridae); note the split posterior end of the cell. **(i)** *L.* cf. *lactosovorans* with a thick flagellum. **(j)** *Leptomonas neopamerae* from *Neopamera* sp. (Heteroptera: Lygaeidae) with a very short flagellum. **(k)** Thick and narrow kDNA disk of *L. acus*. **(l)** Thin and wide kDNA disk of *L. neopamerae*. **(m)** Free flagellum of unnamed strain B05-J13 from the flea *Paraceras melis* (Siphonaptera), collected from the European badger *Melles melles*, and that lacks a paraflagellar rod. **(n)** Free flagellum of *Leptomonas pyrrhocoris* from *Pyrrhocorus apterus* (Heteroptera: Pyrrhocoridae), with an inconspicuous paraflagellar rod (arrowhead). **(o)** Prominent paraflagellar rod (arrowheads) in cross-sectioned flagellum of *L. acus*. **(p)** *Leptomonas podlipaevi* from *Boisea rubrolineata* (Heteroptera: Rhopalidae) with a long flagellum. **(q)** Extremely thin cell of an unnamed strain B09-1267 from the flea *Nycteridopsylla eusarca* (Siphonaptera), collected from the common noctule bat *Nyctalus noctula*. **(r)** Thin promastigote of *L. bifurcata* with a deep flagellar pocket and a kDNA disk bulging out of the cell. **(s)** *Leptomonas wallacei* from *Oncopeltus fasciatus* (Heteroptera: Lygaeidae) carrying a straphanger cyst attached to its flagellum exiting from the flagellar pocket. These flagellates were found in Ecuador, Brazil, Costa Rica and the Czech Republic. Scale bars, 10 μm (a–f), 5 μm (h–j), 2 μm (g, p, q, and s), 1 μm (k, l, and r) and 500 nm (m–o). Figure panels (q) and (s) were kindly provided by E. Suková and M. Attias, respectively. Abbreviations: e, endosymbionts; f, flagellum; ff, free flagellum; fp, flagellar pocket; g, glycosomes; kDNA, kinetoplast DNA; m, mitochondrion; n, nucleus; s, straphanger cyst; sm, subpellicular microtubules.

genetic diversity that might have evolved within the group under the cover of monotonous morphology [17]. For more than a few decades only limited amendments of this system was made through recognition of two additional morphotypes: endomastigotes and opisthomorphs that were used to establish the new genera *Wallaceina* [30] and *Angomonas*, respectively, although the latter genus has been recently redefined phylogenetically ([31] and references therein).

The realization that the morphology- and host-based taxonomy are of very limited value has prompted phylogenetic approaches, initially based on the small subunit ribosomal RNA (SSU rRNA) gene [32–35], and later on the glycosomal glyceraldehyde-3-phosphate dehydrogenase (gGAPDH) gene and other markers [36–38]. These and the subsequent more detailed analyses revealed the major subdivisions within the Trypanosomatidae, although they failed to unequivocally resolve relationships among and within these groups. Even so, the existence of several well-supported clades provided a foundation for phylogenetic redefinition of several genera. Remarkably, the dixenous genera *Trypanosoma* and

Box 2. Potential subfamilies of Trypanosomatidae

(1) The *Trypanosoma* clade. Although this group was thought of as paraphyletic in the past [35,82,83], the more recent data strongly support its monophyly [32–34,36,40,84]. Trypanosomes infect all classes of vertebrates, with predominant vectors being Diptera, Heteroptera, and Siphonaptera [85–87], as well as bloodsucking leeches [88]. The group shows a cosmopolitan distribution [89]. Vector-independent transmission is possible in some cases.

(2) The *Phytomonas* clade. Excluding nonspecific parasites occasionally isolated from plants [42], this is a monophyletic group with specific adaptations to parasitism in plants [45–47]. Transmission occurs by Heteropteran bugs [90,91], distribution is cosmopolitan [28], with a significant economic impact in some regions [91].

(3) The subfamily Leishmaniinae. One of the most species-rich and well-characterized groups of Trypanosomatidae, its members are easily recovered in culture. One of its members, *Crithidia fasciculata*, became a hallmark of insect trypanosomatids, and its genome is now available [92] (http://tritrypdb.org/common/downloads/release-4.1/Cfasciculata).

(4) The *Blastocrithidia* clade. The original taxon under this name has been known since 1959 [4,93], but the phylogeny is unknown for most of the old species, including *Blastocrithidia gerridis*, the type species. Currently, the clade is represented by four named species: *Blastocrithidia triatoma*, *Blastocrithidia leptocoridis*, *Blastocrithidia cyrtomeni*, and *Blastocrithidia largi* [23,58], but also includes numerous unnamed organisms found in the surveys. The clade demonstrates widespread distribution and frequent occurrence in Heteroptera hosts [24,28,74].

(5) The *Leptomonas jaculum* clade. An obscure sister group to the *Blastocrithidia* clade, and is rather obscure [94]. The species that gave its name was not recovered as axenic culture [95]. Few additional members of this clade were found by analysis of gut samples [28,74]. No cultures are currently available. So far it is confined to Heteroptera hosts.

(6) The endosymbiont-bearing clade. This clade is well defined both phylogenetically and by presence of endosymbionts [31,49,96,97]. The original taxonomic affiliations of *Strigomonas oncopelti* (formerly *Crithidia oncopelti), Strigomonas culicis* (formerly *Blastocrithidia culicis*), and *Angomonas deanei* (formerly *Crithidia deanei* and *Herpetomonas roitmani*) were defined by morphology, and this was misleading. The current genus status is based solely on phylogeny [31]. The known members parasitize Diptera and Heteroptera and are easily cultivatable.

(7) The *Herpetomonas* clade. The presence of opisthomastigotes was used to define the original genus [4]. Although this taxon proved to be artificial, several *Herpetomonas* species were found that form a separate clade [32,34,75]. The monophyly was used to redefine the genus regardless of the presence of opisthomastigotes [98]. Its members occur worldwide in Diptera (preferentially), Heteroptera, and Siphonaptera, and are easy to cultivate.

(8) The *Sergeia* clade. *Sergeia podlipaevi* [99] is a member of a clade that appears to be under-represented in current surveys. It is a parasite of biting midges (Diptera: Nematocera) and is available in culture.

(9) The *Leptomonas collosoma* clade. *L. collosoma*, from water bugs (*Gerris dissortis* and *Gerris remigis*) [100], and a group of associated isolates, stand out in the phylogenetic reconstructions [34,39,101–103]. Cultures of these strains are available.

(10–12) Unnamed clades. Trypanosomatids parasitizing Siphonaptera, from the Czech Republic, revealed a novel monophyletic group (clade 10). Members of this group are available in culture. Four members of clade 11 were recently found in surveys of Heteroptera in Southwest Asia and sub-Saharan Africa [28,74]. Five trypanosomatid species parasitizing Brachycera and Heteroptera from Ghana, Kenya, and Macedonia form clade 12. No cultures are available for clades 11 and 12.

Leishmania withstood phylogenetic scrutiny – each genus is now considered monophyletic [33,36,39,40]. Surprisingly, so did the dixenous genus *Phytomonas* that was initially defined primarily by parasitism of plants [41]. This loose criterion, together with frequent (nonspecific or transient) association of diverse trypanosomatids with plants [42], gave justified concerns about the 'arbitrary' nature of this genus [43]. The finding of a clade of plant-associated trypanosomatids (including phloem-, latex-, and fruit-associated parasites), and the realization that other flagellates isolated from plants belong to different groups, represented a *de facto* redefinition of the genus *Phytomonas* in molecular phylogenetic terms [44,45]. In light of findings of the unique mitochondrial metabolism and kinetoplast maxicircle gene organization [46], as well as numerous additional metabolic adaptations to parasitism in plants [47], this group turned out to be rich in molecular synapomorphies that further confirm its status as a natural taxon.

The number of monoxenous species identified by the late 1990s was relatively small, and mainly included organisms that had already been in culture for decades, perhaps reflecting the decline in interest in insect flagellates that were viewed as monotonous and relatively unimportant [48]. However, even with such limited sampling, the artificial status of morphologically defined genera *Crithidia*, *Blastocrithidia*, *Herpetomonas*, and *Leptomonas* became obvious [29]. There were several well-supported major clades or independent deep-branching lineages formed by monoxenous species (Box 2; Figure 2). Among these, only the clade of endosymbiont-bearing Trypanosomatidae has presented an obvious synapomorphy (the presence of endosymbionts) [32,49]. These organisms were previously included in the genera *Crithidia*, *Blastocrithidia*, and *Herpetomonas*, but were reclassified recently as the genera *Angomonas* and *Strigomonas* [31].

Because other monoxenous groups were uncovered solely by molecular phylogenies [39,50], the respective taxa can only be defined in terms of ancestry and descent, a concept which is relatively new to the trypanosomatid field. Although some aspects of the proposed strictly phylogenetic approach to systematics are controversial (in particular, abolishing the Linnean binomial system and obligatory taxonomic ranks) [51], the very idea of defining taxa by phylogeny has gained strength and has been applied to many groups of organisms, including mammals [52–55]. A phylogenetically defined taxon represents a clade that includes a most recent common ancestor and all its descendants ('node-based' definition), or a clade with an attached stem leading to, but not including, the ancestor shared with its sister clade ('stem-based' definition) or an ancestor that first evolved a key (morphological, physiological, etc.) apomorphy, and all its descendants ('apomorphy-based' definition). Each of these definitions is markedly different from the traditional approach to systematics in which the taxa are defined by a set of apomorphies unique to the group ('the traits define the group' [55]). The apomorphy-based definition may appear similar to the traditional approach; however, this phylogenetic approach is primarily based on ancestor–descendent relationships and the choice of traits is secondary ('the group defines the traits' [55]). The choice of the most appropriate definition depends on the available information about a proposed taxon or the biological meaning a taxonomist intends to endow a new taxon with.

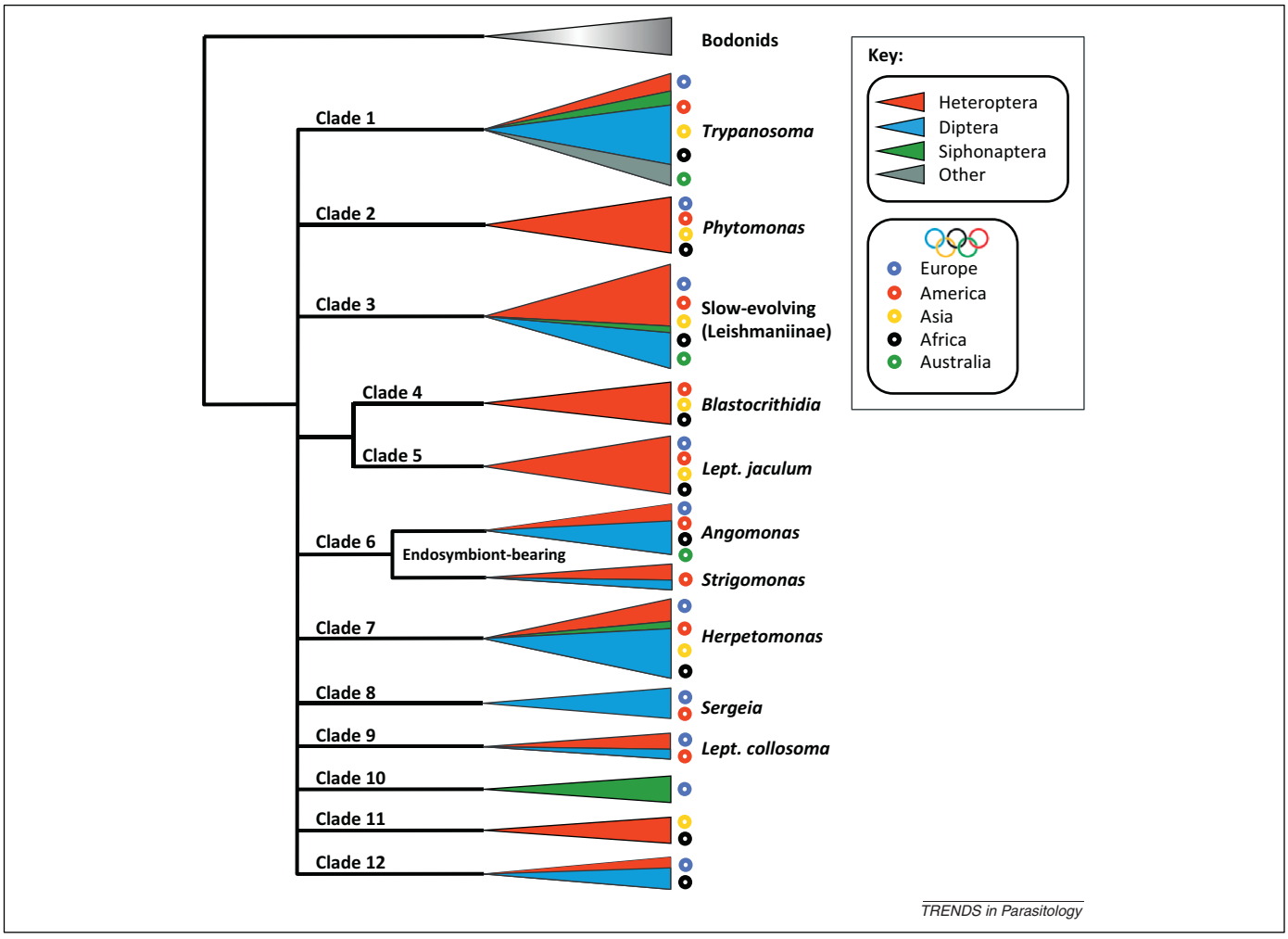

Figure 2. Major clades of the Trypanosomatidae. Evolutionary relationships, host specificity, and geographic distribution among trypanosomatids were inferred from most published reconstructions (see the text), as well as from unpublished results by the authors. The major clades 1 to 12 are described in detail in Box 2. The basal polytomy serves to highlight the fact that relationships among the clades have not been resolved with confidence. The *Trypanosoma* clade often emerges as a sister group to the rest of the family, leaving open an intriguing possibility that the dixenous life cycle of trypanosomes represents an ancestral trait that has been preserved in this lineage but has undergone a reduction in monoxenous parasites. By contrast, the relatively late emergence of *Leishmania* or *Phytomonas* among monoxenous groups testifies to the derived nature of their dixenous life cycles.

Major clades – new subfamilies

The phylogenetic approach has been used recently to establish a new subfamily of Trypanosomatidae. A so-called SE (slow-evolving) clade was characterized by a slower sequence divergence rate of the SSU rRNA genes compared to other trypanosomatid subdivisions [34]. The SE group included not only numerous lineages of presumptively monoxenous trypanosomatids but also the dixenous *Leishmania* that appear relatively late within this clade [34,39,50,56]. Additional monoxenous lineages are likely to be added to this clade in the future. Consistently high phylogenetic support and a clear separation of this clade from the rest of the family indicated that this was a natural taxon, with subfamily being the appropriate rank [57]. The challenge was how to define this taxon. No unifying morphology- or ecology-level traits have so far been found among the members of this group, leaving the phylogenetically relevant nucleotide substitutions the only apomorphies known. This problem was resolved by defining this taxon through the node that represents the most common ancestor and all its descendants [55]. It is most parsimonious to speculate that the ancestor was a monoxenous parasite of the insect intestinal tract. Some adaptations evolved in this organism gave it a selective advantage that allowed rapid expansion of its descendants in various groups of insects. One of the descending lineages, the parasites of ancestral sand flies, had acquired dixeny, giving rise to *Leishmania*. The nature of these adaptations remains elusive but may become obvious by future comparative genomics involving the closest presumptively monoxenous relatives of *Leishmania* – *Leptomonas costaricensis* and *Leptomonas barvae* [39,58]. By uniting a monoxenous ancestor and its mono- and dixenous descendants, the new taxon (subfamily Leishmaniinae) emphasizes the evolutionary origin of the genus *Leishmania*. In this regard, it should be mentioned that the SSU-based and other trees unambiguously showed that the distinction of trypanosomatids into the lower (monoxenous) and higher (dixenous) subgroups [22] used in the old literature is incorrect.

When an evolutionary event at the origin of the clade is known, the apomorphy-based definition of the respective natural taxon can be used [55]. In the case of the aforementioned clade of endosymbiont-bearing trypanosomatids,

such an event can be the acquisition of an endosymbiont by the ancestor of the group [49]. The taxon would then be defined as the original endosymbiont-bearing trypanosomatid (the ancestor of the current genera *Strigomonas* and *Angomonas* [31]) and all of its modern descendants, regardless of the presence of endosymbionts among the latter or other differences among them. The biological meaning of such a taxon would be obvious: this is the group that originated from a specific ancient endosymbiosis event. Again, it should be stressed that the presence of a symbiont would not define this taxon (the group remains to be defined by descent from the specific common ancestor); therefore, descendants rendered endosymbiont-free by the secondary losses would not be excluded. Thus defined, this taxon would not be invalidated if additional endosymbiont-bearing trypanosomatids were discovered that belong to a different clade because those would represent an outcome of a separate endosymbiosis.

More recent extensive sampling of insect trypanosomatids [27,28,34,39,50,58,59] has revealed additional major clades and their number continues to grow. Bootstrap support for most of these is high, and their separation from other clades is wide, suggesting that they were created by some major evolutionary change(s) followed by expansion of the descendant lineages. Even so, in most cases (with a notable exception of the endosymbiont-bearing clade above, and possibly also the clade of *Phytomonas* discussed below) it remains unclear what the nature of that change was, and what distinct biological properties it endowed a clade with. However, the advantage of the strictly phylogenetic approach is that such information is not actually necessary for taxonomic definitions (taxa are not defined by traits) but, if available in the future, can be conveniently added. Trypanosomatidae systematics, therefore, no longer depends on a comprehensive knowledge of the morphological or biochemical traits of a group but only on knowing the phylogeny. A new challenge for a systematist now lies in deciding what level in a branching hierarchy would represent what taxonomic rank. Traditionally, only genus and species ranks have been used in trypanosomatid systematics. Besides the artificial nature of several genera (as discussed above), this rank-poor nomenclature cannot reflect relationships above the genus level. Nor can it be consistently accommodated to multiple branching levels existing in trypanosomatid trees. A radical approach of abolishing traditional ranks seems to be suitable for the complex branching hierarchy observed among the major protistan groups [15]. However, at lower taxonomic levels a potential (and more conservative) solution is to start using taxonomic ranks, such as subfamily and tribe. These taxonomic subdivisions are widely used in other areas of systematics, for example in insects, and are already familiar to most researchers. The major clades of trypanosomatid trees would represent subfamilies – the first among these, the subfamily Leishmaniinae, has been proposed recently [57]. A candidate for the next taxonomic proposal is the clade of endosymbiont-bearing trypanosomatids. In a recent taxonomic revision of this group [31], the clade was subdivided into the genera *Strigomonas* and *Angomonas*, and unification of these genera in a subfamily appears reasonable. Another potential subfamily is represented by the current genus *Phytomonas*, originally defined as dixenous parasites of plants. Although molecular phylogenetic studies showed that most plant trypanosomatids belong to a monophyletic group well-separated from the rest of the family [45], several trypanosomatids with different phylogenetic affinities have also been isolated from plants [60], indicating the artificial nature of the original definition. In addition, distinct subdivisions among the *bona fide* plant parasites have been documented using molecular tools [61–65], justifying subdivision of the entire group into a few new genera. The diversity within other major lineages of Trypanosomatidae has not yet been sufficiently investigated.

Molecular approach to the species problem

The question 'what kind of biological entity constitutes a trypanosomatid species' remains somewhat unclear, just as it does for all organisms with predominantly clonal reproduction (which include many protists) [66]. This conceptual problem is exacerbated by the dearth of stable morphological differences suitable for distinguishing different organisms from each other. Trypanosomatids in culture or within their hosts display a continuum of sizes (Figure 3), and an observed size range often overlaps with other isolates [56,67]. Even when morphological differences are observed, one cannot be certain about their taxonomic value. The long-term adherence of the trypanosomatid systematics to the 'one host – one parasite' concept for species designation was perhaps a silent admission that no better criteria were at hand. As a result, great caution needs to be exercised in evaluating trypanosomatid diversity using traditional morphology-based species descriptions prevailing in the old literature.

A nucleotide sequence-based approach allows substitution of a species with operational proxies: typing units (TUs) delineated in terms of sequence divergence of the

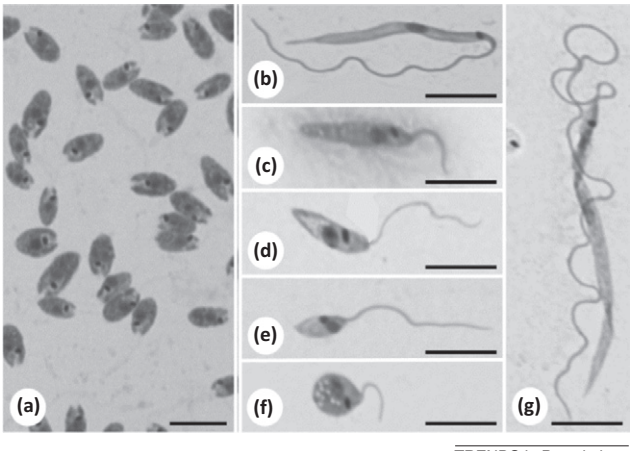

Figure 3. Intraspecific morphological variability. **(a)** Morphologically homogeneous choanomastigotes of *Crithidia inserpata* from *Leptopetalops* sp. (Heteroptera: Coreidae). **(b–g)** Extreme morphological heterogeneity of a strain B07-125 from the flea *Monopsyllus sciurorum* collected from the Eurasian red squirrel *Sciurus vulgaris* (photo by E. Suková). (b) Elongated promastigote with long flagellum. (c) Promastigote with short flagellum. Note the short distance between the kDNA and the nucleus. (d) Short oval promastigote with long flagellum. (e) Choanomastigote with nucleus (arrowhead) more anterior than kDNA (arrow). (f) Spherical round cell with short flagellum. (g) Large elongated promastigote with an extremely long flagellum. Scale bars, 10 μm (a), 5 μm (b–g).

appropriate marker. The relative conservation of the SSU rRNA gene sequences does not provide sufficient resolution among closely related species, as was demonstrated for the species of *Leishmania* [37,39,68] that were established by non-molecular criteria such as differences in natural history, host and vector specificity, and clinical and other data. In a series of works published in the 1990s, the usefulness of kinetoplastid-specific spliced leader (SL) RNA gene repeats was evaluated from the perspective of a phylogenetic marker and a group- or species-specific identification tool [69–71]. The combination of conserved and variable features in this marker enabled development of a PCR-based approach, wherein the conserved exon sequences served as a target for universal primers designed to amplify an entire repeat unit, and the hypervariable intergenic region was used as a molecular marker of high resolution [27,70–73]. The drawback of this marker is that, due to rapid sequence divergence of the intergenic region, a meaningful across-the-family alignment of full-length SL repeat sequences is not possible, leading to a lack of information about long-distance relationships. Nonetheless, a dendrogram depicting the results of a multiple alignment (Figure S1 in the supplementary material online) can reliably demonstrate which sequences are closely related and which are dissimilar. Clusters of repeats defined by the 90% sequence-similarity threshold represent individual TUs. This level was chosen based on the divergence observed in *Leishmania* species, for which abundant additional information is available. Other fast-evolving markers, such as internal transcribed spacer of ribosomal RNA genes, have also been used [31,64].

The SL-based approach was tested in the pilot studies conducted in 2004–2009 in the Neotropics, aimed at the investigation of a segment of the trypanosomatid diversity in the commonly occurring species of Heteroptera in these biodiversity hotspots [27,59]. Subsequently, sampling of flagellates parasitizing Heteroptera has been expanded into Southwest China, Central Europe, the Mediterranean, and equatorial Africa (Table S2 in the supplementary material online) [24,28,74,75].

Most of the organisms were previously unknown, and thus may represent new species. The fact that even small-scale studies in one host taxon (Heteroptera) have resulted in the majority of observed species being new is a reflection of substantial diversity of these flagellates. Its dimensions are difficult to estimate because at this stage we find ourselves at the very start of the species accumulation curve [76]. Moreover, the other major host taxon, Diptera, remains largely uninvestigated, as are other potential host groups, and each is likely to add a new dimension to the world of trypanosomatid diversity.

Due to the preliminary nature of the surveys, only a small number of specimens (usually 1–10) were analyzed from each host species in a given population or locale. This is certainly not enough to detect parasites that occur with low frequency. It is estimated that to detect parasites occurring at 5% prevalence at the 95% confidence level, the sample size must include at least 57 specimens, and if the parasite prevalence is as low as 1% (with the same confidence level), the sample size should be increased to 295 specimens (E. Kozminsky, personal communication). Nevertheless, nearly one third of all analyzed host species were found to harbor trypanosomatids, indicating that parasite prevalence in many populations is higher than 5%.

Concluding remarks

The above description of major clades (Figure 2) will give the reader a flavor of the natural diversity and its poor reflection in traditional trypanosomatid taxonomy. The evidence is abundant that there is no clear correlation between phylogeny and 'classical' morphotypes. However, we also feel that the time is not yet ripe for a major taxonomic overhaul because the diversity of these ubiquitous parasites has so far been uncovered only partially, as is obvious from the recent collections (Box 2). It is reasonable to assume that the new clades will emerge from additional analyses, including deep sequencing of the insect microbiome [77], which will in the future be extended further by including flagellates isolated from other insect hosts, especially Diptera and Siphonaptera.

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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.pt.2012.11.001.

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