Trends in
Parasitology

CellPress

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Hidden passengers: expanding the RNA virosphere of trypanosomatids

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The diversity of trypanosomatid RNA viruses goes far beyond the well-known leishmaniaviruses. Newly discovered viral lineages across multiple families reveal host-specific patterns and distant evolutionary transitions, highlighting the trajectories of viral acquisition and subsequent evolution shaped by parasite biology and ecology.

Diversity and research value of trypanosomatids

Parasitic protists of the family Trypanosomatidae are among the most intensively studied eukaryotic groups, as they include agents of important human diseases such as sleeping sickness, Chagas disease, and leishmaniases. Nevertheless, the diversity of this group extends far beyond those pathogens, encompassing several hundred described species, which differ in the number of hosts required to complete their life cycle: dixenous (involving a vertebrate or plant host and an invertebrate vector) and monoxenous (predominantly confined to insects). Trypanosomatids nonpathogenic to humans often serve as safe models for studying various aspects of the biology of their medically relevant relatives. Moreover, many monoxenous species attract considerable research interest in their own right due to remarkable features such as non-canonical nuclear genetic code, a cell cycle starting with flagellar duplication, and the presence of intracellular bacteria or viruses [1].

RNA viruses in trypanosomatids: the milestones

Trypanosomatids pathogenic to humans were screened for RNA viruses once suitable molecular methods became established. The first viruses, described from *Leishmania* (*Viannia*) *guyanensis* in 1988 and *L*. (*Leishmania*) *major* in 1994, were named LRV1 (*Leishmania* RNA virus 1) and LRV2, respectively. They were classified into the new genus *Leishmaniavirus* within the double-stranded RNA virus family *Totiviridae* [2] (the genus is now in *Pseudototiviridae* [3]).

Interest in trypanosomatid viruses has surged since the discovery in 2011 that their presence can exacerbate the inflammatory response in leishmaniasis [4]. Leishmaniaviruses belonging to the previously characterized LRV1 and LRV2 lineages were identified in several species of the subgenera *Leishmania* and *Viannia* [5]. The virus from *L*. (*Leishmania*) *aethiopica*, discovered in 2014 and initially assigned to LRV2, was recently recognized as a separate species due to sequence divergence and distinct genome structure [3]. Aiming to uncover potential origins of LRVs and inspired by earlier electron microscopy reports of virus-like particles in diverse trypanosomatids [2], researchers expanded screenings to include monoxenous species and dixenous phytomonads (plant parasites). The first such survey in 2018 revealed that diversity of trypanosomatid RNA viruses is not limited to a single genus or family, documenting positive-strand RNA viruses [family *Narnaviridae* and unnamed Tombus-like viruses (TLVs)], negative-strand RNA (family *Leishbuviridae*), and unclassified Ostravirus with unknown strandedness [6]. Twelve new species were described, immediately increasing the known viral spectrum severalfold. Another study published the same year discovered nine new viruses in the flea-dwelling genus *Blechomonas*: three narnaviruses, three leishbuviruses, and, importantly, three leishmaniaviruses [7]. Phylogenetic analysis suggested at least two evolutionary transitions of LRVs between *Leishmania* and *Blechomonas*, while the likely direction – from the former to the latter – was inferred from the parasites' biology. Further sampling of monoxenous trypanosomatids uncovered additional narnaviruses and leishbuviruses, along with new members of *Mitoviridae* (previously regarded as restricted to fungal mitochondria) and recently discovered *Qinviridae* [8–10].

Even virus-free trypanosomatids can retain traces of past viral infections. For example, genomes of *Leptomonas pyrrhocoris* and *Blastocrithidia triatomae* contain 'fossil' sequences similar to those of TLVs [6,10]. Such fragments (termed endogenous viral elements) likely result from retroposition of viral genomic segments, as judged by their proximity to TATE retroelements.

Success with monoxenous trypanosomatids has prompted analysis of previously unscreened dixenous taxa, including neglected subgenera of *Leishmania* – *Mundinia* and *Sauroleishmania* – as well as lizard-parasitic *Trypanosoma platydactyli*, revealing additional members of *Leishbuviridae*, *Narnaviridae*, and *Leishmaniavirus* [11–13]. Even well-studied *Leishmania* (*Viannia*) *braziliensis*, previously known to host only LRVs, proved capable of harboring a leishbuvirus [14].

To date, trypanosomatids have been recorded to host RNA viruses from seven lineages across four phyla of *Orthornavirae* ('true' RNA viruses) and three main strandedness types (Figure 1). Our structure-based phylogeny inference placed the previously orphan Ostravirus in *Reovirales* (Figure 1 and Figures S1–

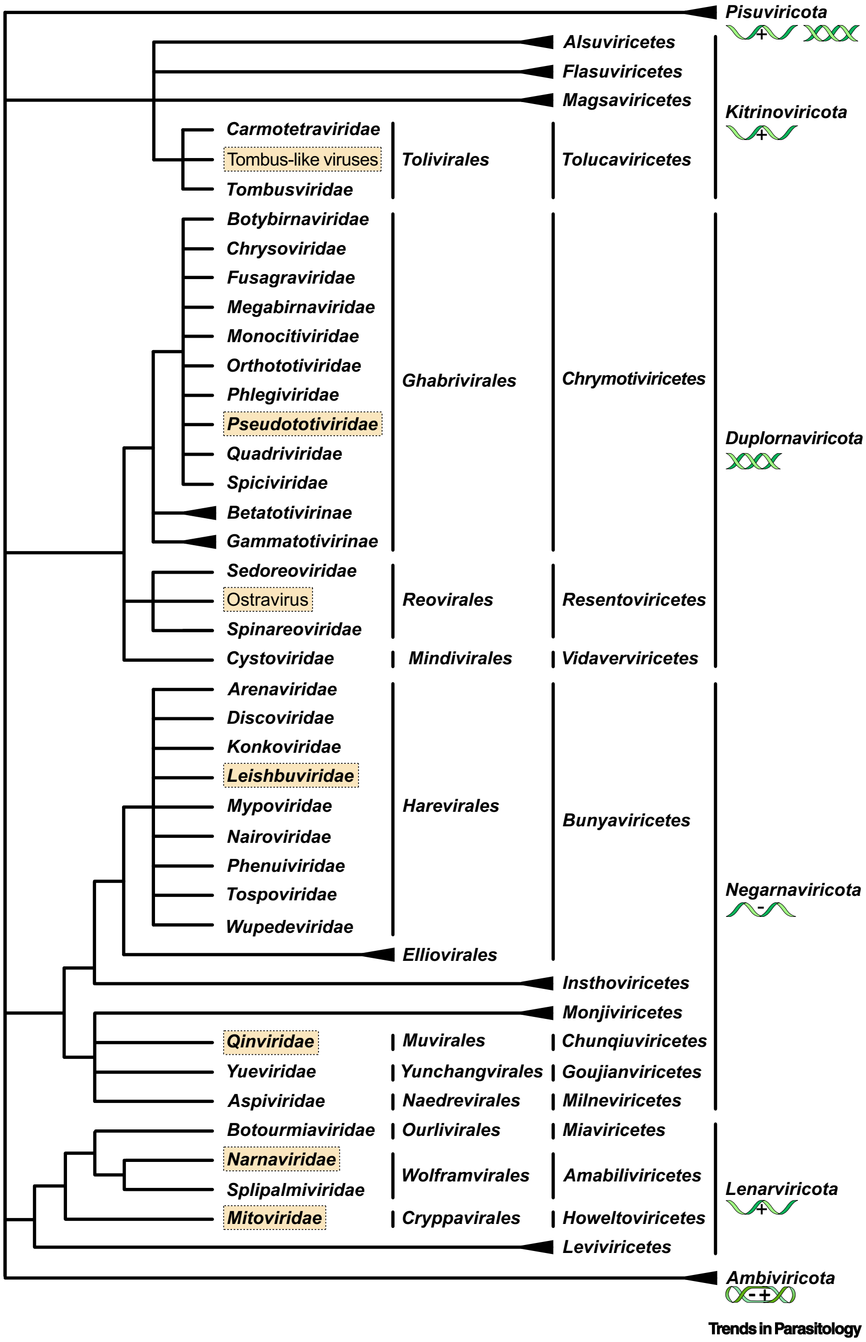
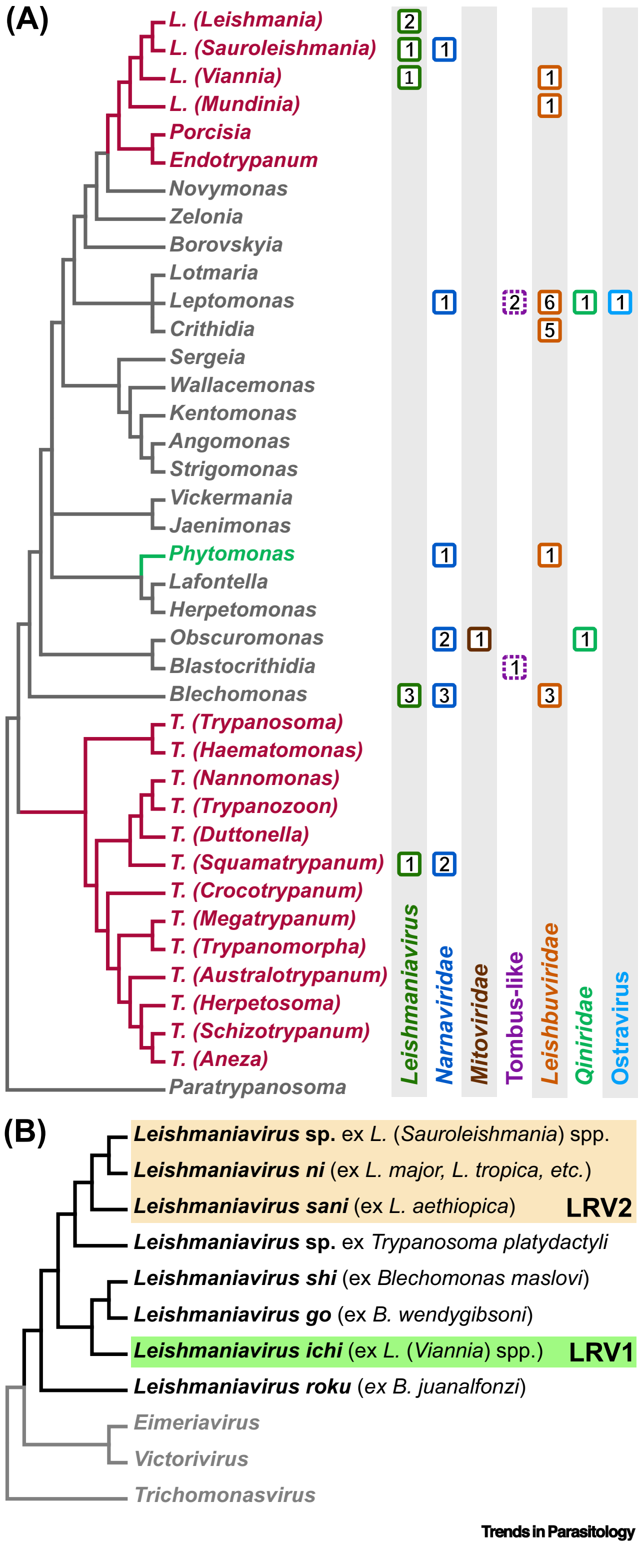


Figure 1. Affiliations of trypanosomatid-infecting RNA viruses. The hierarchical diagram shows the classification of the kingdom *Orthornavirae* according to Virus Taxonomy 2024 Release v.1 [3]. Taxa are ordered from left (families) to right (phyla), with ranks arranged in separate columns (except the suborders *Betatotivirinae* and *Gammatotivirinae*, which are aligned with families for compactness). Trypanosomatid-infecting groups are highlighted, while taxa above the family level that do not contain them are collapsed (triangles). Groups without valid names are shown in regular font. The type of strandedness is indicated symbolically next to the names of phyla.

S4 in the supplementary information online), consistent with its multipartite genome organization and characteristic terminal sequences (see Boxes S1 and S2 in the supplemental information online). Nearly 40 putative viral species are now reported across ten trypanosomatid (sub)genera (Figure 2A), a substantial increase from less than a decade ago, when only LRV1 and LRV2 were recognized. The accelerating rate of virus discovery in these flagellates, alongside numerous unexplored trypanosomatid taxa, indicates a substantial viral diversity yet to be uncovered. The plethora of trypanosomatid cultures available in laboratory collections around the world represent a valuable resource for the discovery of new viruses, whereas the observed general stability of viral infections greatly facilitates this task.

Leishmaniaviruses: what's in the name?

Despite the wide diversity of trypanosomatid viruses currently known, LRVs of *Leishmania* spp. remain the primary focus of research. Numerous studies report the presence of LRVs in specific *Leishmania* isolates or directly in clinical samples from leishmaniasis patients. In most studies, these viruses are referred to as 'LRV1' and 'LRV2' following decades-old nomenclature. However, there are two compelling reasons why this practice should be discontinued. Firstly, 'LRV2' does not correspond to a single taxon (Figure 2B) as viruses from *L. major* and *L. aethiopica* are distinct species [3], while the status of viruses from *Sauroleishmania* spp. warrants further consideration [12]. The LRV from *T. platydactyli*, an undoubtedly distinct species also associated with this lineage (Figure 2B), has not been called LRV2 only due to its recent discovery [13]. Given coevolutionary patterns of LRV1 [5], it may also eventually be split into several species. Secondly, the recent update to viral taxonomy mandates using binomial nomenclature for described species,



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similar to that established for animals, plants, and bacteria [3]. This approach is also better than referring to viruses by their hosts, as, for example, *Leishmaniavirus ni* can infect multiple *Leishmania* spp. [5]. In summary, the growing diversity of known leishmaniaviruses and current nomenclatural rules deprecate using LRV1 and LRV2 as species names, although these abbreviations remain useful for referring to lineages.

Metatranscriptomes: tempting but challenging opportunity

Massively parallel sequencing technologies have become a routine tool for the discovery of RNA viruses, enabling the identification of new taxa in RNA preparations from animals, plants, fungi, protists, and even environmental samples. However, the analyzed specimens often contain multiple organisms, which can obscure the origin of the detected viral sequences. For example, the family *Leishbuviridae* is known from axenic cultures of trypanosomatids and metatranscriptomic samples, most of which originate from insects. Our previous analysis of these metatranscriptomic data revealed that viral sequences were consistently accompanied by trypanosomatid signatures, in line with our view that this family is trypanosomatid-specific [9]. Massively produced metatranscriptomic data from insects (and, potentially, other hosts) provide a promising opportunity to search for new trypanosomatid viruses, overcoming the limitation of culture availability. While this approach is not foolproof even for leishbuviruses, it becomes more challenging for other viral families, for example, *Narnaviridae*, which can infect a wide range of unrelated hosts, including fungi, arthropods, oomycetes, and plants [13]. Therefore, the basis for associating such viruses with trypanosomatids is questionable, and analysis of monospecific laboratory cultures of these organisms remains a gold standard in the field. Nevertheless, as understanding of the diversity of RNA viruses in trypanosomatids advances, this approach may become more justified, following the principle of interpolation. Indeed, it appears that once viruses transition to trypanosomatids, they tend to remain associated with this group, as evidenced by the trypanosomatid-specific genus *Leishmaniavirus*, family *Leishbuviridae*, and some clades within *Narnaviridae* [9,13]. Notably, this does not necessarily imply evolutionary codivergence, as host switches can occur between phylogenetically distant taxa [6,7,9,12].

Viral diversity shaped by available donors

How could the observed diversity of viruses in trypanosomatids have arisen? As suggested by their taxonomic affiliations (Figure 1), these viruses were acquired from various sources, likely at different evolutionary timepoints. Some trypanosomatids are more prone to virus acquisition than others (Figure 2A). This is especially apparent in *Leptomonas pyrrhocoris*, which stands out by hosting seven viral species from four different family-level taxa [9]. The underlying reason for this heterogeneity lies in the host's biology, particularly its feeding behavior, which determines the range of potential viral donors for trypanosomatids. In the case of *L. pyrrhocoris*, its firebug host is omnivorous, exposing its parasites to viruses from plants, invertebrates, fungi, and their associated symbionts or saprotrophs. Conversely, no viruses have been detected so far in the iconic species *Trypanosoma brucei* restricted to hematophagous tsetse flies, which dramatically limits the range of potential viral donors. Notably, dixenous trypanosomatids are unlikely to acquire viruses in vertebrate hosts due to the isolation from other potential viral donors. In insects, trypanosomatids predominantly reside in the gut, where the tight space and high parasite density facilitate frequent interactions with viral donors. These ecological constraints, however, do not act alone, and the flagellates themselves play an important role in shaping patterns of viral acquisition. The presence of several viral lineages confined to trypanosomatids suggests that these parasites are particularly suitable donors for their kin. While frequent co-infections in insects contribute to viral transfer between flagellates, the likely key factor consists in the initial molecular adaptations of a virus to one trypanosomatid species that facilitate exploration of others. Beyond host biology and evolutionary compatibility, population-level factors further influence viral acquisition. The likelihood of such events increases with host/vector abundance and parasite prevalence, as larger populations provide more opportunities for viral transfer. For example, *L. pyrrhocoris* can reach 100% prevalence in its highly abundant and gregarious bug host *Pyrrhocoris apterus* [9].

Taken together, the aforementioned considerations can inform the selection of candidate trypanosomatids for future targeted viral screening.

Figure 2. RNA viruses and their trypanosomatid hosts. (A) Cladogram of Trypanosomatidae showing the distribution of known RNA viruses among genera and subgenera. Phylogenetic relationships are based on the recent family-wide phylogenomic inference [15]. Monoxenous trypanosomatids are shown in grey, while dixenous parasites of vertebrates and plants are shown in crimson and emerald, respectively. Viral presence is indicated by boxes showing the number of documented species. Dotted outlines indicate counts that include a single endogenous viral element. (B) Cladogram of the family *Pseudototiviridae* showing phylogenetic relationships (in agreement with the most recent reconstruction [13]) and coordination of binomial names of *Leishmaniavirus* spp. and their trypanosomatid hosts. Non-trypanosomatid-infecting genera are shown in grey. Lineages corresponding to the obsolete LRV1–LRV2 classification are highlighted.

Acknowledgments

This work was primarily supported by the Czech Grant Agency grant 24-10009S to V.Y. The computational infrastructure used in analyses was purchased in the frame of the EU's Operational Program 'Just Transition' grant LERCO CZ.10.03.01/00/22_003/0000003 to V.Y. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the manuscript writing; or in the decision to publish the results.

Declaration of interests

The authors declare no competing interests.

Supplemental information

Supplemental information associated with this article can be found online at https://doi.org/10.1016/j.pt.2025.12.004.

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https://doi.org/10.1016/j.pt.2025.12.004

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