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Kentomonas gen. n., a New Genus of Endosymbiont-containing Trypanosomatids of Strigomonadinae subfam. n.



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Compared to their relatives, the diversity of endosymbiont-containing Trypanosomatidae remains under-investigated, with only two new species described in the past 25 years, bringing the total to six. The possible reasons for such a poor representation of this group are either their overall scarcity or susceptibility of their symbionts to antibiotics that are traditionally used for cultivation of flagellates. In this work we describe the isolation, cultivation, as well as morphological and molecular characterization of a novel endosymbiont-harboring trypanosomatid species, *Kentomonas sorsogonicus* sp. n. The newly erected genus *Kentomonas* gen. n. shares many common features with the genera *Angomonas* and *Strigomonas*, such as the presence of an extensive system of peripheral mitochondrial branches distorting the corset of subpellicular microtubules, large and loosely packed kinetoplast, and a rudimentary paraflagellar rod. Here we also propose to unite all endosymbiont-bearing trypanosomatids into the new subfamily Strigomonadinae subfam. n.

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Introduction

The taxonomy of the family Trypanosomatidae is in need of a substantial revision to keep pace with the discovery of diversity of kinetoplastid

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flagellates. A handful of erected genera that withstood recent molecular scrutiny includes all the dixenous (employing two hosts in their life cycle) genera *Trypanosoma*, *Leishmania*, and *Phytomonas* (Dollet et al. 2012; Leonard et al. 2011; Lukeš et al. 1997; Schönian et al. 2012). Most of the monoxenous (restricted to a single host) trypanosomatid genera proved to be poly- or paraphyletic (Merzlyak et al. 2001). The traditional classification system, established in the middle of the last century, defined genera on the basis of morphotypes and life cycles, while species were usually delineated based on host specificity (Hoare and Wallace 1966; Vickerman 1976). Since then, new species were being assigned to a given genus based on the combination of described morphotypes (reviewed in Maslov et al. 2013). However, recent results refute such a strict classification, as in many instances, the population of trypanosomatid cells was shown to represent a continuum of several morphotypes and their presence or absence may not correlate with phylogenetic affinity of species (Maslov et al. 2010; Yurchenko et al. 2009; Zídková et al. 2010). The concept of strict host specificity, often referred to as “one host – one parasite” paradigm (Podlipaev 1990), also proved to be either incorrect or of limited application. While some degree of specificity certainly exists – for example, *Leishmania* spp. and *Trypanosoma brucei* are confined to sandflies and tsetse flies, respectively (Bates and Rogers 2004; Holmes 2013), it is certainly not universal. As for the monoxenous trypanosomatids, there are several examples of species with a very narrow range of suitable hosts, illustrated by representatives of the genus *Blechnomonas* restricted to the flea hosts only (Votýpka et al. 2013). Other parasites are less fastidious, as they may parasitize different hosts, sometimes even belonging to different insect orders, such as Heteroptera and Diptera (Týč et al. 2013). One striking example of such loose specificity is the genus *Herpetomonas*. Traditionally restricted to dipteran flies (Borghesan et al. 2013), representatives of this taxon have been found in other insects, plants, and even in ciliates (Fiorini et al. 2001; Fokin et al. 2014).

While the same species of parasite is capable of utilizing different hosts (e.g. *Crithidia brevicula*, Kostygov et al. 2014), the converse applies as well, and the same host and even the same specimen can harbor several trypanosomatid species (e.g. *Pyrrhocoris apterus*, Votýpka et al. 2012). The latter situation is called mixed infection and has been proven to be very widespread (Grybchuk-Ieremenko et al. 2014; Votýpka et al. 2010; Yurchenko et al. 2009). This significantly

complicates species identification and systematics by classical approaches, especially given that sometimes parasites infecting one host species are hardly discernible by morphology (Schmid-Hempel and Tognazzo 2010).

To resolve all these hurdles, an approach employing molecular traits has been proposed (Briones et al. 1992; Teixeira et al. 1997; Yurchenko et al. 2006b). In essence, it relies upon a set of molecular markers that are used to infer phylogenetic relationships between different taxa of Trypanosomatidae. Not surprisingly, given the limitations of the classical approach to systematics discussed above, such molecular approach has proven to be much better suited for the purpose (Jirků et al. 2012; Merzlyak et al. 2001; Teixeira et al. 2011; Zídková et al. 2010).

The latest review of Trypanosomatidae taxonomy considered 13 monophyletic clades at the level of a genus or higher, with 10 being named, formally described, and containing at least one cultivable representative (Maslov et al. 2013). It was argued that this number is not likely to go up significantly, as a fairly comprehensive sampling and analysis of the diversity of monoxenous trypanosomatids in several biological hotspots did not reveal any new taxa on the genus level (Jirků et al. 2012; Votýpka et al. 2010; Westenberger et al. 2004). Currently, seven monophyletic clades represent the monoxenous genera: *Angomonas* and *Strigomonas* characterized by the presence of endosymbionts (Teixeira et al. 2011), *Herpetomonas* and *Sergeia* parasitizing mainly dipteran hosts (Borghesan et al. 2013; Svobodová et al. 2007), *Blastocrithidia*, which is typical for true bugs (Maslov et al. 2013), and recently described genera *Blechnomonas* from fleas (Votýpka et al. 2013) and *Wallacemonas*, usually found in Heteroptera and Diptera (Kostygov et al. 2014; Yurchenko et al. 2014). Three clades are formed by the dixenous genera *Phytomonas*, *Trypanosoma*, and *Leishmania*; however, the monophyletic genus *Leishmania* groups together with the paraphyletic genera *Leptomonas* and *Crithidia* within the monophyletic subfamily Leishmaniinae (Jirků et al. 2012). Finally, three remaining clades were so far recognized on the basis of environmental samples only (Maslov et al. 2013). One of these formally undescribed clades, first observed in the biodiversity analysis of trypanosomatid parasites of Brachycera flies (clade 1 in Týč et al. 2013), deserves special attention. The initial results strongly suggested that this monophyletic group, composed of closely related typing units TU116 and TU117, differs from the other members of the family Trypanosomatidae to an extent that justifies establishing a

new genus-level taxon. In the 18S rRNA-based phylogenetic tree it formed a sister group to the *Angomonas/Strigomonas* clade. Nevertheless, the absence of a cultured representative precluded its more detailed study and consequently its formal description.

The *Angomonas/Strigomonas* clade unites two trypanosomatid genera harboring endosymbiotic bacteria (Teixeira et al. 2011). It has been long known that insect-infecting trypanosomatids harbor bacterial endosymbionts of the genus *Candidatus Kinetoplastibacterium*. In bacterial nomenclature, *Candidatus* is a component of the taxonomic name for a bacterium that cannot be maintained in a bacteriology culture collection. It is an interim taxonomic status for non-cultivable organisms. The first endosymbiont was recognized more than one hundred years ago as diplosoemes in *Strigomonas culicis* (Novy et al. 1907). Fifty years later similar structures were described in *S. oncopelti* as bipolar bodies (Newton and Horne 1957).

The endosymbiosis in trypanosomatids is a mutualistic relationship, which resembles an early stage of organelle acquisition. It is characterized by an intensive metabolic exchange (d'Avila-Levy et al. 2005a, b; Kořený et al. 2010). Bacteria usually maintain those genes that are necessary for the biosynthesis of compounds essential for their hosts, exemplified by enzymes and metabolic precursors completing indispensable biosynthetic pathways of the protist, such as those for amino acid, lipid and purine/pyrimidine metabolism (Motta et al. 2013). This explains the low requirement for these elements, such as hemin and vitamin B12, in endosymbiont-harboring trypanosomatids (Chang et al. 1975; Granick and Sassa 1971). The genomic content of these bacteria is highly reduced, indicating that the cooperation between endosymbionts and their hosts is complemented by multiple horizontal gene transfers from bacterial lineages to trypanosomatid nucleus. Importantly, such transfers preferentially occurred in parts of the pathways that are missing from other eukaryotes (Alves et al. 2013a, b).

Endosymbionts found in both trypanosomatid genera described to date are similar, being classified in the β -division of Proteobacteria, and all attempts for their cultivation outside their host failed. Recent phylogenetic analysis places proteobacterial endosymbionts of trypanosomatids within the Alcaligenaceae family, as a sister group to *Achromobacter* and *Bordetella*, and divided them into two clades reflecting the taxonomy and phylogeny of their hosts from the genera *Angomonas* and

Strigomonas (Alves et al. 2013a; Teixeira et al. 2011).

The bacterium is closely associated with the host's cell nucleus and is usually surrounded by glycosomes. It divides in a coordinated manner with other host cell structures before the basal body and kinetoplast segregations, thus ensuring that each daughter cell inherits a single bacterium (Motta et al. 2010). However, the presence of β -proteobacteria is not the only common feature of the genera *Angomonas* and *Strigomonas*, which also share several unique ultrastructural traits. The most relevant are the differences related to the cytoskeleton, kinetoplast and paraflagellar rod: (i) the subpellicular microtubules are absent in sites where the mitochondrial branches are juxtaposed to the plasma membrane, (ii) the kinetoplast is large with a relatively loose network of kinetoplast (k) DNA fibrils, and (iii) the cryptic paraflagellar rod (PFR) lacks one of its critical component, PFR2 (Freytmüller and Camargo 1981; Gadelha et al. 2005). The extended mitochondrion may signify an increased respiratory demand and metabolic rate (Fenchel 2014). Within the kinetoplast, DNA and basic proteins are distributed not only in the kDNA network, but also in the kinetoflagellar zone, a region between the kDNA and the inner mitochondrial membrane proximal to the flagellum (Cavalcanti et al. 2008). Importantly, the removal of the endosymbiotic bacterium did not affect the host cell morphology (Freytmüller and Camargo 1981). On the other hand, an artificial aposymbiotic strain established by prolonged chloramphenicol treatment was unable to colonize insects. This implies that endosymbiotic bacteria influence the protist cell surface composition and, consequently, the parasite's ability to bind to the insect midgut (Catta-Preta et al. 2013).

In addition to bacteria, several Trypanosomatidae species were documented to harbor virus-like particles (de Souza and Motta 1999; Yurchenko et al. 2014). The biological significance of this phenomenon is not well understood at the moment, but one can hypothesize that the double stranded (ds) RNA viruses alter the transcriptional profile of the host cell, giving it a selective advantage over its virus-free counterparts. This situation has been reported in several *Leishmania* species where virus-containing isolates were more pathogenic to humans (Ives et al. 2011; Zangger et al. 2014).

In this work we describe the isolation, cultivation, and morphological and molecular characterization of a novel endosymbiont-harboring trypanosomatid, *Kentomonas sorsogonicus* sp. n. To accommodate this species, a new genus *Kentomonas*

gen. n. is being erected that shares many common features with the genera *Angomonas* and *Strigomonas*, but also differs from them in some important details. Here we also propose to unite all endosymbiont-bearing trypanosomatids into a new subfamily Strigomonadinae subfam. n.

Results

Isolation, Primary Characterization and Subcloning of a New Trypanosomatid Species

The *Sarcophaga* (sensu lato; family Sarcophagidae) sp. female fly (collection # M57) was captured on April 2nd, 2013 in the vicinity of Donsol, Sorsogon, the Philippines (12°54'40"N; 123°35'28"E; 4 m a.s.l.). The infection with trypanosomatid-like cells was localized to the hindgut. The environmental (= env) and cultured isolates were named MF-08-env and MF-08, respectively. Two environmental samples belonging to the TUs 116 and 117 (Ecu-07-env and Ecu-06-env, respectively, see below) also used in this work were described elsewhere (Týč et al. 2013). They originated from flies of the families Sarcophagidae (genus *Ravinia*) and Lauxaniidae, respectively, and were captured in Ecuador in 2008.

The environmental and cultured isolates (MF-08/MF-08-env) were first characterized in molecular terms by sequencing their 18S rRNA gene. Their sequences turned out to be 100% similar (GenBank Acc. No. KM242075), confirming the identity of the cultured isolate. They were also highly similar but not identical to the previously characterized 18S rRNA gene from the Ecu-06-env isolate (TU117, GenBank Acc. No. KC206002). This isolate along with another Ecuadorian isolate Ecu-07-env (TU116), was previously identified as a member of the proposed new taxon (Týč et al. 2013). Therefore, we decided to characterize the first cultured isolate belonging to this clade in more detail.

We generated two clonal lines (MF-08.01 and MF-08.02) and compared them side-by-side with the original MF-08 isolate by 18S rRNA sequencing. All lines were identical to both environmental and cultured isolates of MF-08 (GenBank Acc. No. KM242075), and one clone (MF-08.01) has been chosen for all subsequent analyses. Both original isolate and clonal lines can be cultivated in media without FBS. In this case, cells become immobile and adhere to the plastic surface of the cultivation flask. Such a phenotype can be reversed by the addition of 10% FBS (data not shown).

Morphological and Ultrastructural Characterization

Light microscopic examination of MF-08.01 revealed several species- (or group)-specific features. All cells were barleycorn-shaped with various alternative positions of kinetoplast, ranging from the typical choanomastigotes to opisthomorphs (Fig. 1A-D) (Maslov et al. 2013; Merzlyak et al. 2001; Yurchenko et al. 2006b). Their size ranged from 6.2 to 10.2 μm ($8.0 \pm 0.8 \mu\text{m}$) and from 1.9 to 3.7 μm ($2.6 \pm 0.3 \mu\text{m}$) in length and width, respectively. The flagella varied in length from 7.2

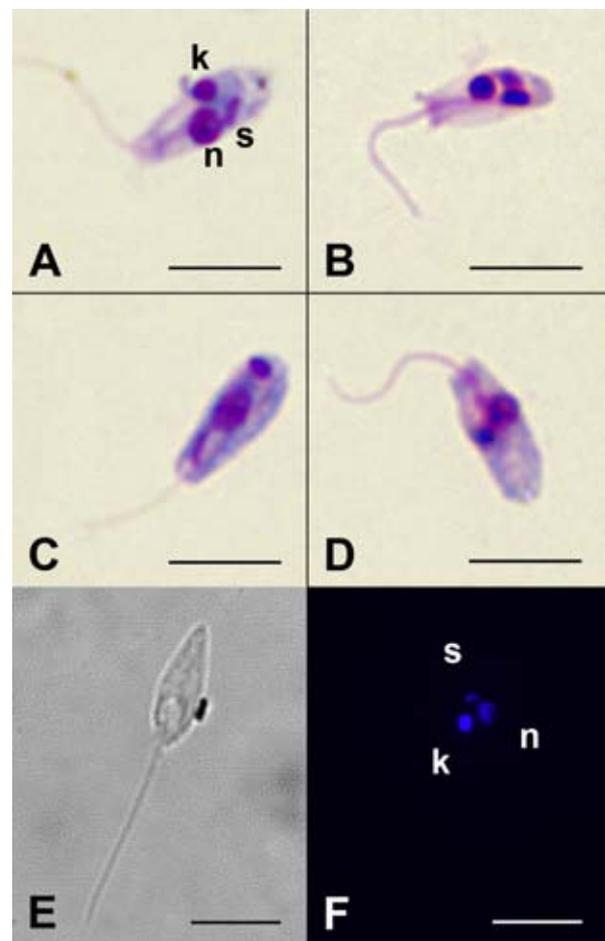


Figure 1. Light microscopy of *Kentomonas sorsogonicus* sp. n. (clone MF-08.01). **A-D**, Giemsa-stained choanomastigotes with various relative positions of kinetoplast (k) and nucleus (n) are shown. Endosymbiotic bacteria (s) can be detected in the cytoplasm. Light (**E**) and fluorescent (**F**) microscopy of the DAPI-stained *K. sorsogonicus* reveal the same features. Scale bars are 5 μm .

to $12.8\ \mu\text{m}$ ($9.8 \pm 1.3\ \mu\text{m}$). Importantly, the MF-08.01 cells were morphologically indistinguishable from the cells observed *in situ*. Light and fluorescent (DAPI) microscopy detected rod-shaped endosymbiotic bacteria inside the trypanosomatid cell (Fig. 1; s – symbiont).

Next, the MF-08.01 cells were analyzed by SEM (Fig. 2A) and HPF-TEM (Fig. 2B-F). We recently demonstrated that the HPF protocol improves fine structure of the trypanosomatid cells (Yurchenko et al. 2014). Our SEM analysis confirmed that the MF-08.01 cells were typical blunt-ended choanomastigotes with well-developed pellicular ridges and a relatively long flagellum. The opening of the flagellar pocket is edged by a distinct ring (Fig. 2A), and when exiting the pocket, the flagellum becomes widened (Fig. 2B). HPF-TEM revealed all the typical trypanosomatid features such as oval nucleus, basal bodies, glycosomes, electron-dense kinetoplast disc within a reticulated mitochondrion rich with tubular cristae. On the other hand, the following unique or discriminating traits were observed: (i) Kinetoplast was of the cylindrical shape, contained a loose network of kDNA fibrils packed in parallel to the axis of the disk (Fig. 2C) and measured between 395 and 778 nm in thickness ($538 \pm 75\ \text{nm}$; $N=25$) and between 347 and 524 nm in diameter ($437 \pm 43\ \text{nm}$; $N=25$); (ii) The extensively branched mitochondrion penetrates to the periphery reaching the pellicle (Fig. 2D), where it disrupts the corset of subpellicular microtubules and forms ridges well-visible by TEM (Fig. 2D) and SEM (Fig. 2A); (iii) A single endosymbiont is present (Fig. 2B); (iv) Two or three rows of desmosomes attach the flagellum to the membrane of the flagellar pocket (arrowheads in Fig. 2B and E); (v) The paraflagellar rod is inconspicuous but some thin electron-dense structure is present instead (Fig. 2F).

Phylogenetic Analyses

The gGAPDH, 18S rRNA and SL RNA genes were amplified and sequenced as described elsewhere (Yurchenko et al. 2006a). The 18S rRNA gene was 99% and 92% homologous to Ecu-07-env and Ecu-06-env sequences (GenBank Acc. Nos. KC206002 and KC206003) corresponding to TU116 and TU117, respectively. These so far stand-alone Ecuadorian isolates (Týč et al. 2013) constituted a well-supported clade including MF-08.01, with Ecu-07-env sequence being its closest relative.

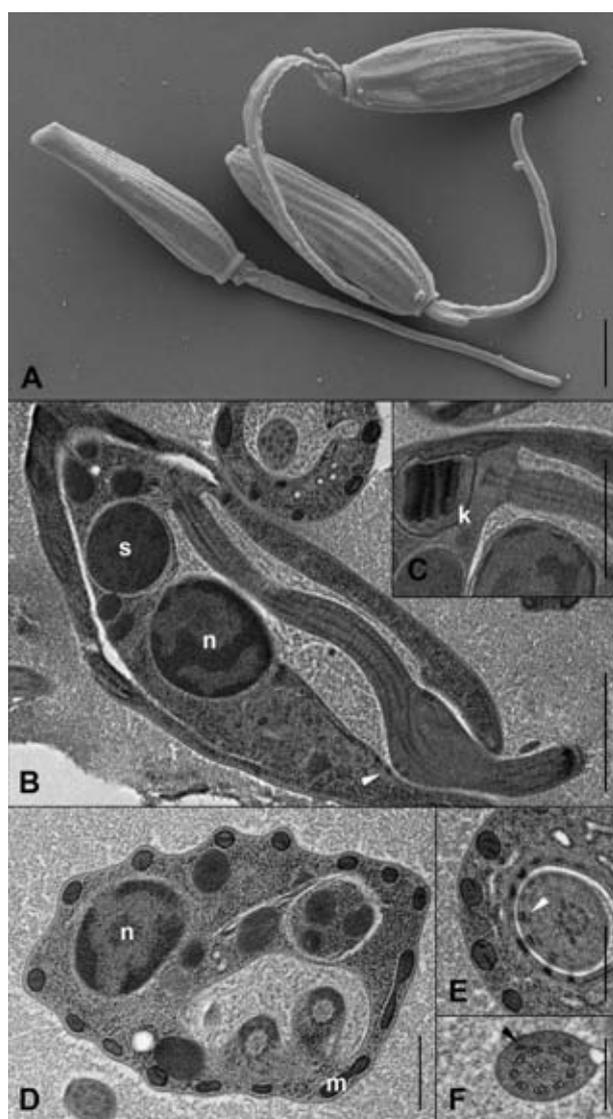


Figure 2. Scanning (A) and high-pressure freezing transmission (B-F) electron microscopy of *Kentomonas sorsogonicus* sp. n. (B, C) – longitudinal sections showing typical features of trypanosomatids such as nucleus (n) and kinetoplast (k) as well as the bacterial symbiont (s). White arrowhead demonstrates several rows of desmosomes in the contact area between flagellum and the membrane of the flagellar pocket. (D) – cross-section of the cell displays extended mitochondrion (m) reaching plasma membrane and breaching the layer of subpellicular microtubules. (E) – cross-section of the flagellum at the opening of the flagellar pocket. Desmosomes are marked by white arrowhead. (F) – cross-section of the free flagellum. Black arrowhead indicates rudimentary paraflagellar rod. Scale bars are $2\ \mu\text{m}$ (A), $1\ \mu\text{m}$ (B, C), $500\ \text{nm}$ (D, E) and $250\ \text{nm}$ (F).

The gGAPDH gene of MF-08.01 was not as conserved and exhibited only 91% similarity to its *Crithidia brachyflagelli*, *Leptomonas spiculata* and *Leptomonas acus* orthologs (Jirků et al. 2012; Yurchenko et al. 2008). We have also amplified gGAPDH from Ecu-06-env (TU117, GenBank Acc. No. KM242074), which shared 91% homology and clustered with MF-08.01 (data not shown). We were not able to amplify the gGAPDH gene of Ecu-07-env (TU116). The sequence repeatedly recovered in our analysis (GenBank Acc. Nos. KM242073) belonged to *Angomonas desouzai*. As demonstrated before, Ecu-07-env has originated from a mixed infection with at least three different Trypanosomatidae species belonging to TU110, TU116, and *A. desouzai* (Týč et al. 2013).

The SL RNA gene is a marker most suitable for resolving relationships between closely related species/sub-species/populations of insect Trypanosomatidae (Westenberger et al. 2004; Yurchenko et al. 2006b). Similarly to the gGAPDH analysis presented above, in the SL RNA-based comparison, the closest relative of MF-08.01 was Ecu-06-env (TU117) (GenBank Acc. Nos. KM242076 and KM242077). They shared only 49% identity, clearly indicating that these two isolates belong to different species, but clustered together on the SL RNA-based dendrogram (data not shown). As for TU116, we again failed to amplify its SL RNA gene. All obtained sequences from Ecu-07-env (GenBank Acc. Nos. KM242078 and KM242079) showed low similarity with the SL RNA genes from *Wallacemonas* spp. and environmental trypanosomatid isolate of *Drosophila melanogaster* (Wilfert et al. 2011; Yurchenko et al. 2014). This confirms our observation that TU116 represented only a minor fraction of this environmental isolate.

For phylogenetic reconstruction, 18S rRNA sequences were aligned with a set representing major trypanosomatid clades. The most optimal Bayesian and maximum likelihood trees were congruent and consistent with previously published ones (Fig. 3). The well-supported monophyletic group recovered in our analysis consisted of MF-08.01, Ecu-06-env (TU117), and Ecu-07-env (TU116) and constituted a sister branch to the *Angomonas/Strigomonas* clade that unites flagellates harboring bacterial endosymbionts (Teixeira et al. 2011). The monophyly assessment under the maximum likelihood criterion showed that the optimal topology has the highest value in the approximately unbiased test (Supplementary Material Fig. S1). Meanwhile several other topologies that do not contain a clade of

endosymbiont-bearing trypanosomatids could not be excluded. Interestingly, the representatives of the new group formed the longest branches within Trypanosomatidae. It can be explained by many substitutions in nucleotide positions being conserved in other members of the family (data not shown) resulting in relatively low statistical support of this clade in the ML analysis – the long-branch attraction effect. In Bayesian inference the posterior probability for the group increased significantly when covarion model was applied (from 0.73 to 0.99). This method was specially designed for cases when conservative regions start to evolve rapidly (Tuffley and Steel 1998). The Bayes factor topology test under the covarion model showed “very strong” evidence ($2 \log_e = 33.48$) for the monophyly of endosymbiont-containing Trypanosomatidae (Kass and Raftery 1995).

The phylogenetic trees inferred using gGAPDH gene sequences were incongruent to those discussed above (Supplementary Material Fig. S2). Therefore despite the established tradition, we did not concatenate 18S rRNA and gGAPDH gene sequences. All genera of endosymbiont-harboring trypanosomatids - *Angomonas*, *Kentomonas*, and *Strigomonas* - formed well-supported and clearly distinct clades with different phylogenetic affinities on the tree. *Kentomonas* formed a sister group to Leishmaniinae, while *Angomonas* and *Strigomonas* appeared to branch earlier (Supplementary Material Fig. S2). The gGAPDH sequence is known to be more susceptible to the change in evolutionary rate that leads to dramatic disturbance of topology (Zidková et al. 2010). In addition, we also demonstrated a bias in nucleotide distribution of gGAPDH sequences. The third position is heavily predisposed to contain G or C nucleotides, with GC content varying between approximately 60% in *Trypanosoma* spp. to 95% in Leishmaniinae (Supplementary Material Fig. S3 and table 3 in Hannaert et al. 1998). We also tried to use first two codon nucleotides only to infer relationships among Trypanosomatidae, but the resulting tree showed a significant decrease in phylogenetic resolution (data not shown).

To confirm the presence and phylogenetic position of symbiotic bacteria in MF-08.01 (Figs 1 and 2), 16S rRNA gene and the ITS region between 16S and 23S were amplified and sequenced (GenBank Acc. Nos. KM242070 and KM242071). Phylogenetic analysis reliably placed this bacterium, named here *Candidatus Kinetoplastibacterium sorsogonicusi*, within the group of other β -proteobacteria encountered in trypanosomatids (Fig. 4). The species at the base of the

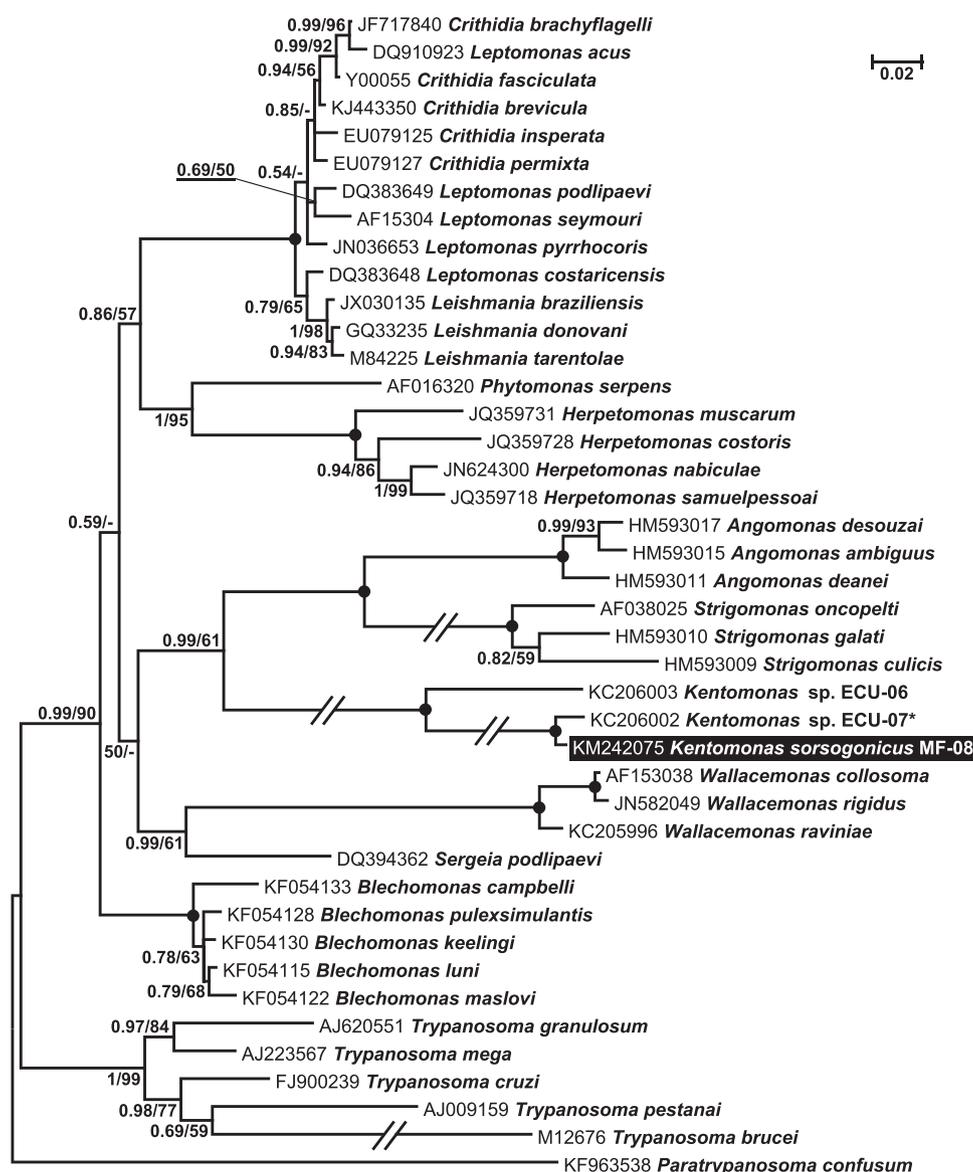


Figure 3. 18S rRNA-based Bayesian phylogenetic tree of Trypanosomatidae. Names of species for sequences retrieved from GenBank are indicated. Species newly described in this work is highlighted. Bootstrap values from Bayesian posterior probabilities (5 million generations) and bootstrap percentage for maximum likelihood analysis (1,000 replicates) are shown at the nodes. Dashes indicate bootstrap support below 50% or different topology. Black dots represent 100% bootstrap support and Bayesian posterior probability of 1.0. Double-crossed branches are at 50% of their original lengths. The asterisk indicates a mixed infection sample. The tree was rooted with *Paratrypanosoma confusum* sequence. The scale bar denotes the number of substitutions per site.

clade were endosymbionts of *Angomonas* spp., but these relationships were poorly supported.

Viruses

Viruses or viral-like particles were detected in several endosymbiont-bearing species of the

Angomonas/*Strigomonas* clade (Motta et al. 2003; Soares et al. 1989; Teixeira et al. 2011). To clarify whether this trait is species-specific or it is a feature of the whole group, we analyzed MF-08.01 for the presence of viruses. All the viruses of Trypanosomatids described to date belong to the diverse group of dsRNA viruses. Virus-like

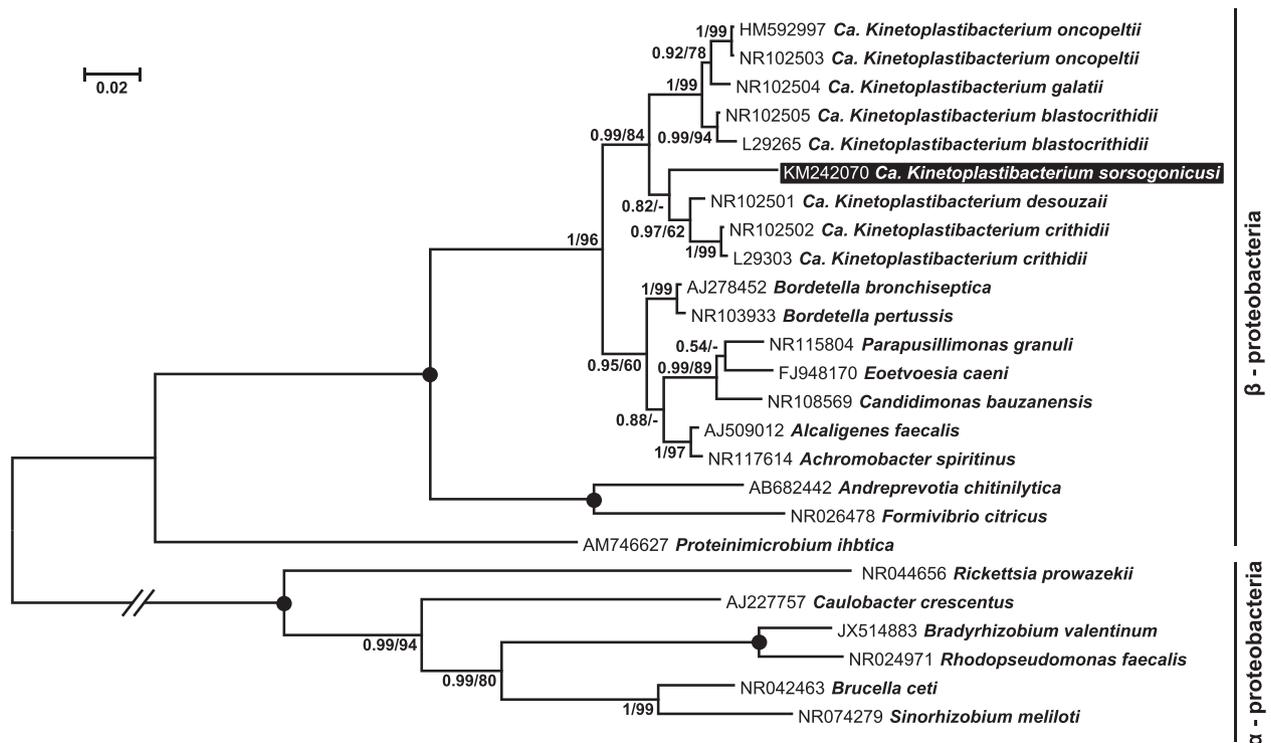


Figure 4. 16S rRNA-based Bayesian phylogenetic tree of bacterial endosymbionts of trypanosomatids. Names of species for sequences retrieved from GenBank are indicated. Species newly described in this work is highlighted. Bayesian posterior probabilities (5 million generations) and bootstrap percentage for maximum likelihood analysis (1,000 replicates) are shown at the nodes. Dashes indicate bootstrap support below 50% or different ML topology. Black dots represent 100% bootstrap support and Bayesian posterior probability of 1.0. The tree was rooted with sequences of 6 α -proteobacteria species. The scale bar denotes the number of substitutions per site.

particles were detected neither by immunofluorescent microscopy using anti-dsRNA antibodies (data not shown), nor in total RNA samples digested with DNase and S1 nuclease, leaving dsRNA intact (Supplementary Material Fig. S4).

Discussion

A recent overview of the known diversity of trypanosomatids led to the conclusion that all or at least most major clades of these morphologically rather uniform flagellates have already been discovered (Maslov et al. 2013), although surprising findings still occur. For example, a novel genus *Paratrypanosoma* has been recently discovered in the gut of *Culex pipiens* mosquitoes. Phylogenomic analyses showed this genus to constitute a distinct clade between the free-living bodonids and the obligatory parasites represented by the genus *Trypanosoma* and other Trypanosomatidae

(Flegontov et al. 2013). Another fascinating group is represented by two sister genera *Angomonas* and *Strigomonas* that are united by the presence of symbiotic β -proteobacteria *Kinetoplastibacterium* spp. in their cytoplasm (Motta et al. 2010, 2013; Teixeira et al. 2011). It is highly likely that this intimate relationship initiated by a random acquisition of a bacterium appeared relatively late in the trypanosomatid evolution, as it is present only in a single clade that was for a long time considered to be confined to South America only (Teixeira et al. 2011), although it was recently also reported from other continents (Týč et al. 2013). Studies of this eukaryote-prokaryote relationship revealed that *Kinetoplastibacterium* spp. synthesizes heme and provides it to its heme-auxotrophic host (Alves et al. 2011; Chang et al. 1975; Kořený et al. 2010). Indeed, this may actually be one of the few metabolites (along with some essential amino acids, vitamins and cofactors) that makes the β -proteobacterium

indispensable (Alves et al. 2013a; Klein et al. 2013).

To date there were only six described species of endosymbiont-bearing trypanosomatids and only two of them were isolated within the last 25 years (Teixeira et al. 2011). This may be due to overall rarity and modest diversity of these flagellates. Nevertheless there is another possible reason for this phenomenon. The generally applied protocol for isolating new strains of insect trypanosomatids relies on the extensive usage of antibiotics that keeps the contaminating bacteria at bay but at the same time kills endosymbiont-bearing trypanosomatids (Chang 1974). Interestingly, in the course of its introduction into the culture, MF-08.01 also went through a period when it was exposed to a panel of antibiotics including bactericidal amikacin, penicillin, and chloramphenicol, yet its establishment was successful, the culture became axenic and the protist proved to be able to retain its endosymbiont.

Representatives of the genus *Kentomonas* are widely distributed (so far found in Ecuador and the Philippines) and parasitize brachyceran flies of the families Lauxaniidae and Sarcophagidae, which are commonly known as flesh flies. They are ovoviviparous and often deposit their hatched or hatching maggots on feces, decaying material or open wounds of mammals. This behavior makes horizontal transmission of parasites rather straightforward. Importantly, one of the *Kentomonas*-bound environmental samples (Ecu-07) was found as a component of a mixed infection along with other monoxenous species of genera *Angomonas* and *Wallacemonas*.

Thanks to this cultivable representative, we herein formally describe a well-defined group that has been previously identified on the basis of environmental samples only and hence not suited for a formal taxonomic recognition (Týč et al. 2013). This clade is clearly different from the related genera *Angomonas* and *Strigomonas* both phylogenetically and morphologically (e.g. characteristic ridges on the cell surface), yet shares with them the distinctive endosymbiont. To accommodate MF-08.01, we propose to establish *Kentomonas* gen. n., and a new subfamily Strigomonadinae, with *Kinetoplastibacterium* presence in the cytoplasm being its main synapomorphy (Teixeira et al. 2011). The newly erected subfamily is a well-defined monophyletic group equipped with a set of unifying traits, such as the large loosely packed kinetoplast, rudimentary or absent paraflagellar rod, and highly branched mitochondrion that extends to the plasma membrane, where it breaches the corset of subpellicular microtubules.

Taxonomic Summary

Class Kinetoplastea Honigberg, 1963 emend. Vickerman, 1976

Subclass Metakinetoplastina Vickerman, 2004

Order Trypanosomatida Kent, 1880

Family Trypanosomatidae Doflein, 1901

Subfamily Strigomonadinae Votýpka, Yurchenko, Kostygov et Lukeš, 2014

Diagnosis: A clade of Trypanosomatidae defined by the following apomorphic traits: 1) presence of the endosymbiotic bacteria; 2) well developed system of the peripheral mitochondrial branches disrupting subpellicular layer of microtubules; 3) large and loosely packed kinetoplast; 4) rudimentary paraflagellar rod.

Etymology: The name of the subfamily has originated from the name of the first described genus of this clade, *Strigomonas* (Lwoff and Lwoff 1931).

Genus *Kentomonas* Votýpka, Yurchenko, Kostygov et Lukeš, 2014

Generic diagnosis: A well-supported monophyletic group of monoxenous trypanosomatids of invertebrate hosts (e.g. Diptera: Sarcophagidae, Lauxaniidae) harboring bacterial endosymbionts. It is defined by a set of unique sequences of the 18S rRNA, gGAPDH and SL RNA genes. Molecular phylogenetic analyses confirm this genus as a member of the family Trypanosomatidae; however, it cannot be associated with any valid genus.

Etymology: The generic name honors William Saville-Kent, an English protistologist. In the monograph "A manual of the Infusoria: including the description of all known flagellate, ciliate, and tentaculiferous Protozoa, British and foreign, and an account of the organization and affinities of the sponges" published between 1880 and 1882 he erected the first two genera of monoxenous trypanosomatids, *Herpetomonas* and *Leptomonas*, and placed them together with the genus *Trypanosoma* into the new order Trypanosomata (now Trypanosomatida). "monas" (Greek) – monad; third declension (*monas*); feminine; the word *monas* is included in many generic names of flagellates.

Kentomonas sorsogonicus Votýpka et Lukeš sp. n.

Fig 1-2.

Species diagnosis and description: Cultured *Kentomonas sorsogonicus* cells are of the typical choanomastigote morphology with various positions of the kinetoplast and characteristic ridges on the cell surface. Cells range from 6.2 and 10.2 μm in length and between 1.9 and 3.7 μm in width, with flagellum measuring from 7.2 to 12.8 μm . Branches of the mitochondrion press on the plasmatic membrane from the inside that results in formation of the ridges on the cell surface. The kinetoplast disk is loosely packed and varies between 395 and 778 nm in thickness, 347 to 524 nm in diameter. Endosymbiotic β -proteobacteria are present in the cytoplasm. The species is identified by the unique sequences KM242075 (18S rRNA), KM242072 (gGAPDH) and KM242076 (SL RNA), and belongs to TU 172.

Type host: *Sarcophaga (sensu lato)* sp. (Diptera: Brachycera: Sarcophagidae), female. The xenotype (2013/F-MF08) is deposited at Charles University, Prague.

Site: Intestine (hindgut).

Type locality: Vicinity of Donsol, Sorsogon, the Philippines (12°54'40"N; 123°35'28"E; 4 m a.s.l.).

Type material: Hapantotype (Giemsa-stained slide 2013/F-MF-08/S), axenic culture of the primary isolate (MF-08) and clonal lines (MF-08.01 and MF-08.02) are deposited in the research collections of respective

institutions in Prague, Ostrava and České Budějovice, Czech Republic.

Etymology: The species name is derived from the name of city (Sorsogon) where type locality is situated.

Remarks: Based on the sequences of 18S rRNA (KC206002 and KC206003), gGAPDH (KM242073 and KM242074), and SL RNA (KM242077, KM242078, and KM242079), the environmental samples from flies captured in Ecuador (Otongatchi), Ecu-06-env (TU117) of the family Lauxaniidae and Ecu-07-env (TU116) of the genus *Ravinia* (Sarcophagidae), also belong to the genus *Kentomonas*.

***Kentomonas sorsogonicus* endosymbiont:** “*Candidatus Kinetoplastibacterium sorsogonicus*” Yurchenko et Kostygov sp. n.

Type material: Obligate symbiotic β -proteobacteria in the cytoplasm of *Kentomonas sorsogonicus*.

Diagnosis: The species is identified by the unique sequences KM242070 (16S rRNA) and KM242071 (ITS rDNA).

Etymology: “*sorsogonicus*” refers to the name of the trypanosomatid host species.

Methods

Parasite isolation and establishing of cultures and clonal lines: Insects were collected, dissected and examined under a microscope as described previously (Maslov et al. 2007; Votýpka et al. 2012; Yurchenko et al. 2009). To establish the primary cultures, contents of the insect intestines were cultivated in the Brain Heart Infusion (BHI) medium (Sigma-Aldrich, St. Louis, USA) supplemented with 10 μ g/ml of hemin (Jena Bioscience GmbH, Jena, Germany), 10% Fetal Bovine Serum (FBS), 500 units/ml of penicillin, 10 μ g/ml of chloramphenicol, 10 μ g/ml of amikacin, 10 μ g/ml of 5-fluorocytosine and 0.5 mg/ml of streptomycin. In all the following subcultures only amikacin was used. Several independent clonal lines were established by plating multiple serial dilutions of the primary culture onto a 1% agar medium supplemented with BHI and antibiotics as described earlier (Kostygov et al. 2011; Popp and Lattorf 2011). The identity of clonal lines was confirmed by sequencing their 18S rRNA gene (see below). Obtained cultures and clonal lines were deposited in the collections of the Department of Parasitology, Charles University, Prague, in the Life Science Research Centre of the University of Ostrava, and in the Institute of Parasitology, České Budějovice, Czech Republic and are available upon request.

Light and electron microscopy: Light microscopy of Giemsa or 4',6-diamidino-2-phenylindole (DAPI) stained smears was done as described elsewhere (Yurchenko et al. 2006b). Standard measurements were performed for 50 cells on Giemsa-stained smears and expressed in micrometers (μ m). For scanning electron microscopy (SEM), cultured cells were fixed in 2.5% (v/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.2). Following transfer to the poly-L-lysine-coated cover slips, post-fixation in 2% OsO₄ in 0.1 M phosphate buffer for 2 hrs, dehydration in an ascending acetone series, critical point-drying with CO₂ in Pelco CPD2 (Ted Pella Inc., Redding, USA), and sputtering with gold in a Sputter Coater Polaron chamber (Polaron Ltd., Watford, UK), the samples were observed using a JEOL 7401-F microscope (Jeol Europe, Prague, Czech Republic) at accelerating voltage of 80 kV. High pressure freezing transmission electron microscopy (HPF-TEM) was performed essentially as described elsewhere (Yurchenko et al. 2014) with the following modifications: after

HPF, samples were substituted with the frozen substitution medium (2% OsO₄ in 100% acetone) pre-cooled to -90 °C. The temperature profiles and incubation timings were the same as before. Images were captured using an Orius SC1000 CCD camera (Gatan, München, Germany).

PCR amplification, cloning and sequencing: Total genomic DNA was isolated from 10 ml of the axenically grown cultures using High Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol. 18S rRNA gene was amplified from 10 to 100 ng of total genomic DNA using primers S762 and S763 (Maslov et al. 1996), cloned and sequenced. Genes for glycosomal glyceraldehyde-3-phosphate dehydrogenase (gGAPDH) and SL-RNA were amplified using primer pairs M200 – M201 and M167 – M168, respectively (Maslov et al. 2010; Westenberger et al. 2004).

To amplify the complete 16S rRNA sequence of the bacterial endosymbiont, the primers P1seq and 1486R were used (Teixeira et al. 2011). The internal transcribed spacer (ITS) region between 16S and 23S rRNA genes was amplified with primers P3Seq and P23sRev (Du et al. 1994). All PCR products were cloned and sequenced as described above.

The GenBank accession numbers for the new sequences determined in the course of this work are KM242075 (18S, MF-08.01), KM242072 (gGAPDH, MF-08.01), KM242074 (gGAPDH, Ecu-06-env), KM242073 (gGAPDH, Ecu-07-env, *A. desouzai*), KM242076 (SL, MF-08.01), KM242077 (SL, Ecu-06-env), KM242078 (SL, Ecu-07-env, var. 1), KM242079 (SL, Ecu-07-env, var. 2), KM242070 (16S, *Ca. Kinetoplastibacterium sorsogonicus*), KM242071 (16S-ITS-23S, *Ca. K. sorsogonicus*).

Phylogenetic analyses: 18S rRNA gene sequences of trypanosomatids were aligned using Muscle 3.8.3.1 (Edgar 2004) and the resulting alignment was manually refined using BioEdit v 7.2.5 (Hall 1999) and ambiguously aligned positions were removed. Final dataset contained 42 taxa and 2,005 nucleotide positions. Evolutionary model (GTR+I+G) was selected using Akaike criterion in jModeltest 2.1.4 (Darriba et al. 2012). Maximum likelihood phylogenetic inference was performed in RAxML v 8.0 with the selected model and 1,000 “thorough” bootstrap replicates (Stamatakis 2014). The monophyly testing was performed using AU (Approximately Unbiased) test in CONSEL v 0.1j software with site likelihoods calculated in RAxML (Shimodaira and Hasegawa 2001). The optimal topology was rated with those found in bootstrap replicas. Bayesian inference was accomplished in MrBayes 3.2.2 with analysis run for 5 million generations under GTR+I+G model (5 gamma categories) with covarion and sampling every 1,000 generations (Ronquist et al. 2012). Other parameters were left in their default states. The hypothesis of *Angomonas* + *Strigomonas* + *Kentomonas* monophyly was tested using Bayes factors with marginal likelihoods estimated using stepping-stone method (100,000 generations).

gGAPDH gene sequences were aligned using Muscle 3.8.3.1 as above and the resulting alignment was checked by eye to prevent artifactual frameshifts. ML analysis and model testing were performed with the use of Treefinder (v. 03.2011). Comparison of different partitioning schemes using AIC and BIC showed substantial advantage of separating model parameters for the three codon positions. The selected substitution models (according to AIC) were as follows: GTR+G (1st and 2nd pos.), J3(= TIM1)+G (3rd pos.) with 5 gamma categories in each case. Statistical support of bipartitions was assessed with the use of bootstrap resampling (1,000 replicas). Bayesian inference of phylogeny was accomplished in MrBayes 3.2.2 with analysis run for 5 million generations, sampling every

1,000 generation and other parameters of MCMC set as default (Ronquist et al. 2012). GTR+G with 5 gamma categories was used for each of the three codon positions with all model parameters and rate multiplier unlinked.

Reconstruction of phylogeny of endosymbionts was performed in a similar way with few differences specified below. Since the alignment of 16S rRNA gene sequences was more accurate, no positions were removed from the alignment. The dataset contained 25 taxa and 1,576 nucleotide positions. The evolutionary model selected by jModeltest was TIM2+I+G and therefore PhyML 3.0 (Guindon et al. 2010) was used for phylogenetic inference under maximum likelihood criterion. Heuristic search was performed using the SPR branch swapping algorithm. In Bayesian analysis covarion model was not applied. All accession numbers of the sequences used in these analyses are listed on the respective phylogenetic trees (Figs 3 and 4).

Detection of dsRNA viruses: For detection of dsRNA viruses, two complementary protocols were used. Cells were stained with mouse monoclonal anti-dsRNA (Scicons, Szirák, Hungary) followed by goat anti-mouse IgG –Alexa Fluor 488 (Life Technologies, Carlsbad, USA) antibodies as described previously (Zangger et al. 2013). In addition, 50 µg of total RNA isolated using TRI reagent (Sigma-Aldrich) was treated with 1 unit of DNase I (New England Biolabs, Ipswich, USA) at 37 °C for 1 hr, followed by digestion with 35 units of S1 nuclease (Sigma-Aldrich) for 45 min at the same temperature. Samples were analyzed on 0.8% native agarose in 1xTAE buffer (Beiting et al. 2014).

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Appendix A. Supplementary Data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.protis.2014.09.002>.

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