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Characterization of a new cosmopolitan genus of trypanosomatid parasites, *Obscuromonas* gen. nov. (Blastocrithidiinae subfam. nov.)

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Abstract

The expanding phylogenetic tree of trypanosomatid flagellates (Kinetoplastea: Trypanosomatidae) contains a long-known and phylogenetically well-supported species-rich lineage that was provisionally named as the '*jaculum*' clade. Its members were found in representatives of several unrelated families of heteropteran bugs captured in South and Central America, Europe, Africa, and Asia. However, this group resisted introduction into the culture, a needed prerequisite for its proper characterization. Here we describe four new cultivable species, which parasitize various parts of their hosts' intestine, including the thoracic and abdominal part of the midgut, hindgut, and Malpighian tubules. Morphologically, the cultured flagellates vary from relatively short stumpy promastigotes to long slender leptomonad cells. Some species form straphangers (cyst-like amastigotes) both *in vivo* and *in vitro*, initially attached to the basal part of the flagellum of the mother cell, from which they subsequently detach. To formally classify this enigmatic monophyletic cosmopolitan clade, we erected *Obscuromonas* gen. nov., including five species: *O. modryi* sp. nov. (isolated from the true bug host species *Riptortus linearis* captured in the Philippines), *O. volfi* sp. nov. (from *Catorhintha selector*, Curaçao), *O. eliasi* sp. nov. (from *Graptostethus servus*, Papua New Guinea), *O. oborniki* sp. nov. (from *Aspilocoryphus unimaculatus*, Madagascar), and *O. jaculum* comb. nov. (from *Nepa cinerea*, France). *Obscuromonas* along with the genus *Blastocrithidia* belongs to the newly established Blastocrithidiinae subfam. nov.

Keywords: Biodiversity; Cyst-forming trypanosomatids; Monoxenous kientoplastids; Parasites; Phylogeny; Ultrastructure

Introduction

Trypanosomatid flagellates are well-known as the causative agents of serious human, animal, and plant diseases, but increasingly also as diverse and speciose parasites of insects (Lukeš et al. 2018). Since insects have the largest biomass of all terrestrial organisms, with the estimated number of ~10 quintillions of individuals alive at any moment, their parasites must be also comparably numerous (Stevens 2001). With the estimated worldwide prevalence of trypanosomatid flagellates exceeding 10% of all dipteran and heteropteran hosts (Maslov et al. 2013; Lukeš et al. 2018), and taking into account the fact that each infected insect con-

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tains thousands of these protists, their total number must be truly staggering.

Within the last two decades, morphology was replaced by molecular phylogeny as the decisive criterion for the establishment of new protist species, as well as their higher taxonomic ranks (d'Avila-Levy et al. 2016; Votýpka et al. 2015; Adl et al. 2019). In trypanosomatids, the 18S rRNA-based phylogenies are frequently extended by the spliced-leader RNA and glycosomal glycerol-3-phosphate dehydrogenase (gGAPDH) genes, allowing fine-tuning of the evolutionary relationships (d'Avila-Levy et al. 2015; Lukeš et al. 2018).

Members of the genera *Trypanosoma* and *Leishmania* are, due to their pathogenicity for humans, intensely studied and, as a consequence, their diversity, distribution, and host specificity are known in minute details (Maslov et al. 2019), while only fragmentary and often not connected pieces of data were collected for all other trypanosomatid genera. However, collections of flagellates isolated from insects across all continents except Antarctica allowed a rather comprehensive mapping of the diversity, prevalence, and host-parasite relationships of these omnipresent protists (Wallace 1966; Podlipaev 1990; Votýpka et al. 2012a; Schwarz et al. 2015; Borghesan et al. 2018; Králová et al. 2019). While the diversity of insect-dwelling trypanosomatids is growing with every study, there are indications that we may be reaching saturation and that all major lineages are already represented in the available data. Although the trypanosomatid systematics and taxonomy remained very conservative till the end of the last century (Podlipaev 1990; Votýpka et al. 2015), the recently described diversity of hundreds of typing units (TUs, proxies of species) resulted in the establishment of 10 new genera (*Blechomonas*, *Borovskyia*, *Jaenimonas*, *Kentomonas*, *Lafontella*, *Lotmaria*, *Novymonas*, *Paratrypanosoma*, *Wallacemonas*, and *Zelonia*) (Flegontov et al. 2013; Votýpka et al. 2013, 2014; Kostygov et al. 2014, 2016; Hamilton et al. 2015; Schwarz et al. 2015; Yurchenko et al. 2016; Kostygov and Yurchenko 2017; Espinosa et al. 2018). Two other well-supported clades were recalcitrant to cultivation, preventing their formal taxonomic establishment. One of them, previously known as "clade II *sensu* Týč et al. 2013" was recently described and named *Vickermania* (Kostygov et al. re-submitted), while the other one is the subject of this study.

A recent extensive examination of the dipteran and heteropteran hosts in the tropical forests of Papua New Guinea allowed the identification of dozens of putative novel species (Králová et al. 2019), yet no deeper-branching clade was recovered that would justify the description of a new genus. The analyses of extensive sets of TUs from other diversity hotspots (Borghesan et al. 2018; Votýpka et al. 2019, 2020) led to the same conclusion. Hence, while there is still massive unmapped diversity of trypanosomatids in the insect hosts, this diversity occurs below the genus level and it is unlikely that there exist many undescribed monophyletic clades on the levels of a genus and higher. It also appears that the diversity of insect trypanosomatids is not as overwhelming as predicted previously (Podlipaev 2001; Stevens 2001) since a substantial fraction of the described TUs parasitizes more than one host species (Lukeš et al. 2018).

However, phylogenetic trees mapping the diversity of the expanding dataset of monoxenous trypanosomatids constantly expose one clade that remained either unnamed or was provisionally labeled as '*jaculum*' (Týč et al. 2013; Votýpka et al. 2013; Votýpka et al. 2019, 2020; Králová et al. 2019). This designation is derived from the species *Leptomonas jaculum*, inhabiting the intestine of the water scorpion *Nepa cinerea* (Heteroptera). The 18S rRNA-based phylogenetic analysis revealed that this trypanosomatid is closely related to the genus *Blastocrithidia* and the length of its branch is clearly on par with that of other genera (Kostygov and Frolov 2007). However, it was proposed that a higher taxonomic unit should be established only when several conditions are fulfilled, one of which is the availability of at least one representative TU in the culture, allowing a future in-depth study (Votýpka et al. 2015). Since we managed to introduce six different strains (four distinct TUs) that unambiguously branch within the '*jaculum*' clade into the axenic cultures, this important requirement has now been fulfilled, allowing us to establish a new genus *Obscuromonas* gen. nov.

Materials and methods

Fieldwork, cultivation, and host identification

Trypanosomatid TUs described here were collected in the course of several expeditions. The first one was to Madagascar in March 2010, where bugs were sampled in Ambatofosty (19°16′46.79″S, 47°28′44.47″E). Additional samples were collected in Papua New Guinea in May 2011, at the New Guinea Binatang Research Centre in Nagada (5°9′23″S, 145°47′41″E). The third expedition was to the Philippines in March 2013, where insects infected with trypanosomatids of the '*jaculum*' clade have been sampled in Bontoc (17°5′58″N, 120°59′22″E). Finally, a subset of trypanosomatid flagellates described here originates from the Caribbean island of Curaçao (12°10′45.09″N, 68°57′54.28″W) visited in April 2015.

In frame of these expeditions, insects have been collected by using sweep netting, handpicking, CDC light traps, or collection from an illuminated white blanket. The insects were dissected within 12 h of their capture, and the infected tissues were processed for DNA, smears, and cultivation following a protocol described elsewhere (Lukeš and Votýpka 2020). Upon transport into the laboratory, in some cases, it took several months before the axenic culture could be established.

The digestive tract was removed in a way that did not compromise the insect except a few abdominal segments. The species identity of the dry- or wet-mounted specimens has been established by Petr Kment (Department of Entomol-

ogy, National Museum, Prague, Czech Republic), where the specimens have also been deposited.

DNA extraction, PCR amplification, sequencing, and phylogenetic analysis

Based on our previous studies (Votýpka et al. 2019, 2020), 18S rRNA sequences of three cultivable strains belonging to two TUs are already available in the GenBank database: M-09 (TU***88***; acc. no. MT174475), M-12 (TU***88b***; acc. no.MT174476), and CC-37 (TU***236***; acc. no. MK056199). 18S rRNA sequences of the other three cultivable strains (PNG-17, PNG-74, and Fi-14) have been obtained as described previously (Votýpka et al. 2010). The newly obtained sequences showed 100% identity with the already existing sequences (Králová et al. 2019), and, thus, do not represent new TUs.

Alignments for phylogenetic analyses were generated by MAFFT v.7 (Katoh and Standley 2013), with ambiguously aligned positions in the trimmed alignment being removed manually in Geneious software v.10.0.6 (https://www.geneious.com). All the available sequences in GenBank of the (nearly) full-size 18S rRNA gene from trypanosomatids, except the intensely studied genera *Trypanosoma* and *Leishmania* (that were represented by just a subset of species), were used. The final dataset contained 404 taxa and 2,488 characters. Analyses were performed in PhyML v.3.0.1 (Guidon and Gascuel 2003) and MrBayes v.3.2.2 (Ronquist et al. 2012) with model optimization in ModelTest v.3.06. A general time-reversible substitution model with a mixed model for among site rate variation (GTR + Γ + I) was chosen as the best fitting model of sequence evolution. Bootstrap analyses involved heuristic searches with 1,000 replicates (Maximum likelihood). Bayesian inference analysis was run for five million generations with covarion and sampling every 100 generations. All other parameters were left in their default states.

Light and transmission electron microscopy

For light microscopy of Giemsa or 4′,6-diamidino-2-phenylindole (DAPI)-stained smears on poly-L-lysine coated slides was using an Olympus BX51 microscope equipped with a DP71 charge-coupled device (CCD) camera (Olympus, Tokyo, Japan). Standard measurements were performed for 50 cells of each morphotype on Giemsa-stained smears and expressed in micrometers (μm). For scanning electron microscopy (SEM), cultured cells were fixed in 2.5% (vol/vol) glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) and processed as described previously (Yurchenko et al. 2014).

Samples were observed using a JEOL JSM-7401-F microscope (JEOL, Tokyo, Japan) with an accelerating voltage of 4 kV. High-pressure freezing followed by transmission electron microscopy (HPF-TEM) was performed essentially as described in Yurchenko et al. (2014). Images were captured on a JEOL JEM-1010 microscope (JEOL) using a Mega View III camera (EMSIS GmbH, Münster, Germany) and measurement (in nanometers, nm) of kinetoplasts for 30 cells were performed as described previously (Lukeš and Votýpka 2000).

Results and discussion

Isolation of new trypanosomatid species

In the course of expeditions to Madagascar, Papua New Guinea, the Philippines, and Curaçao, hundreds of heteropterans and dipterans were dissected and examined by light microscopy for the presence of trypanosomatid flagellates. If these have been detected in any compartment of the intestinal tract, including Malpighial tubules, the samples have been processed as described elsewhere (Lukeš and Votýpka 2020).

In all cases, we have tried to establish primo-cultures from flagellates found in the infected heteropteran bugs, which proved more challenging for members of the '*jaculum*' clade than for other trypanosomatids. There are several reasons for that. Firstly, due to the exceptionally slow growth of the '*jaculum*' species, in mixed infections they become invariably overgrown by other parasites. Secondly, in contrast to other trypanosomatids, the '*jaculum*' species cannot be established from isolated primary cultures, which are always to some extent contaminated by fungi, yeast, and/or bacteria, since the traditional axenization methods (Lukeš and Votýpka 2020) cannot be applied to such slowly growing protists. Finally, the '*jaculum*' species are finicky when the growth conditions are concerned, even in non-contaminated single infections.

Although their establishment in culture is challenging and requires months of dedicated cultivation efforts, we eventually succeeded with six strains representing four different TUs branching in the '*jaculum*' clade and established their axenic cryo-preservable stocks (Table 1). Cultures in flat flasks were maintained at 23 °C. In most cases, we have used the following conditions: biphasic solid blood agar (2% agar, 2% neopeptone, 0.6% NaCl, and 20% of rabbit or ovine blood) with the liquid phase composed by Brain Heart Infusion (BHI supplemented with 10 mg/mL hemin), RPMI 1640 (with HEPES), Complete Schneider's (Drosophila; Insect) Medium, and M 199 Medium (1:1:1:1) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and antibiotics (100 μg/mL amikacin or gentamicin). The exact growth kinetics was not studied; however, in the initial passages, when the starting density was about 10^5 parasites per ml, it took three to five weeks to reach the density of about 5×10^6. After many months of continuous growth, in some cases (Fi-14, M-09, and M-12) the cultures have been passaged on a weekly basis, with the densities reaching up to 5×10^7 parasites per ml.

It is probably just a coincidence that all established axenic cultures come from the above-mentioned islands, and none

Table 1. Summarized information about the newly described species of the genus *Obscuromonas* gen. nov.: strain; typing units (TUs); true bug (Heteroptera) host species and family; intensity (int.) of parasite infection; localization (site) of the infection in the host intestine (AMG – abdominal midgut, HG – hindgut, MG – midgut, MT – Malpighian tubules, TMG – thoracic midgut); the predominant morphotype; place and date of the host capture.

	Strain	TU	Host species	Family	Int.	Site	Morphotype	Country	Locality	Date
O. modryi	Fi-14	TU***6/7C***	*Riptortus linearis*	Alydidae	4	MG	Promastigotes (+straphangers)	The Philippines	Bontoc	26.03.2013
	PNG-17		*Riptortus linearis*	Alydidae	3	MG	Promastigotes	Papua New Guinea	Nagada	04.05.2011
O. oborniki	M-09	TU***88***	*Aspilocoryphus unimaculatus*	Lygaeidae	3	AMG, TMG	Long leptomonades	Madagascar	Ambatofosty	12.03.2010
	M-12	TU***88b***	*Spilostethus pandurus*							
O. volfi	CC-37A	TU***236***	*Catorhintha selector*	Coreidae	3	HG, MG	Promastigotes	Curaçao	Souax	18.04.2015
O. eliasi	PNG-74	TU***187***	*Graptostethus servus*	Lygaeidae	2	MT	Promastigotes	Papua New Guinea	Nagada	09.05.2011

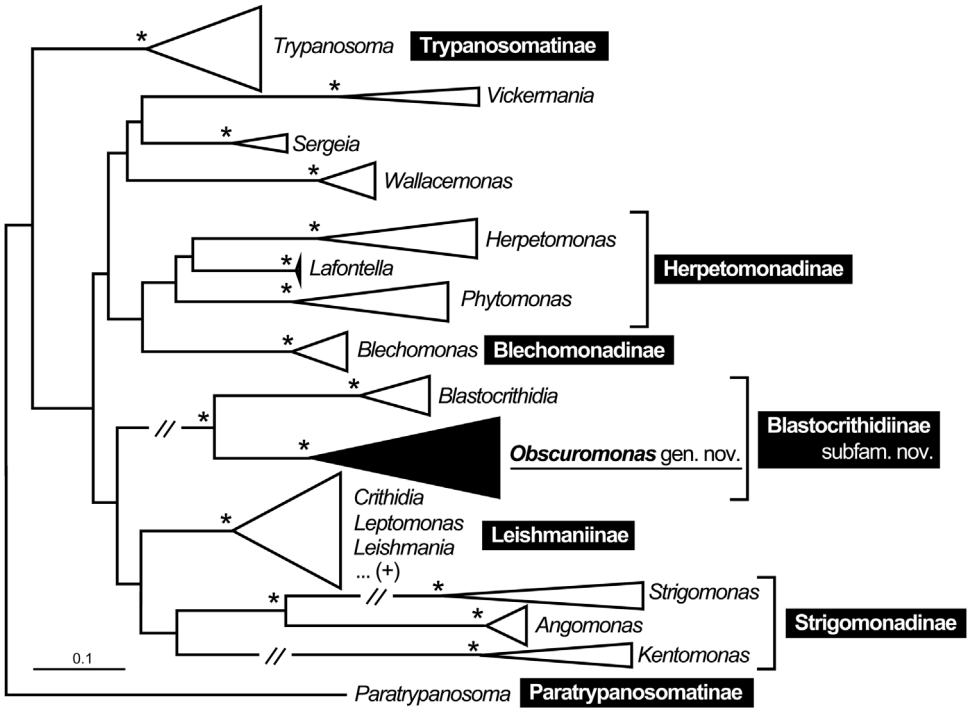

Fig. 1. Phylogenetic tree of the family Trypanosomatidae based on the 18S rRNA gene sequences and reconstructed using the Maximum likelihood method. Branches are collapsed at the generic level, showing mutual relationships among genera and the higher-order taxonomy. Asterisks mark branches with maximal statistical support (bootstrap values for maximum likelihood >90, Bayesian posterior probabilities >0.95); double-crossed branches are 50% of the original length; the scale bar denotes the number of substitutions per site. (+) the subfamily Leishmaniinae includes the monoxenous genera *Leptomonas*, *Crithidia*, *Lotmaria*, *Novymonas*, *Zelonia*, and *Borovskyia* and the dixenous genera *Leishmania* (including four subgenera *Leishmania*, *Viannia*, *Sauroleishmania*, and *Mundinia*), *Porcisia*, and *Endotrypanum.*

from several expeditions carried out on the mainland (*e.g.*, Central and Southern Europe, South-East Asia, central and South America) (Lukeš et al. 2018, Suppl. information). For the lists of dissected insect hosts and detected TUs in these habitats see our previous studies (Králová et al. 2019; Votýpka et al. 2019, 2020).

One of the most common TU within the '*jaculum*' clade (Fig. 2) is TU***6/7C***, which is represented in our subset by two strains established in the culture: the Papua New Guinean strain PNG-17 isolated from *Riptortus linearis* (Alydidae) and the Philippine strain Fi-14 isolated from the same host species. Both infections were very strong, with parasites localized in the midgut of their hosts. The observed dominant morphotype was a middle-sized promastigote. The infections were monospecific. In the case of the strain Fi-14, we noted the presence of straphangers sporadically attached to some cells. The typing unit TU***88*** is also represented by two axenic strains, both from Madagascar: M-09 from *Aspilocoryphus unimaculatus* and M-12 from *Spilostethus pandurus* (both host species belong to the family Lygaeidae). Both strains occupy the abdominal as well as thoracic part of the midgut and long slender leptomonad cells were observed at high density. TU***187*** is represented by a single Papua New Guinean strain PNG-74 isolated from *Graptostethus servus* (Lygaeidae). Moderate infection of middle-sized promastigotes was observed exclusively in the Malpighian tubules. The last trypanosomatid species represented by TU***236*** was isolated in Curaçao from *Catorhintha selector* (Coreidae), where the middle-sized promastigotes were abundantly present in both the abdominal midgut and hindgut.

Phylogenetic analyses

Phylogenetic tree based on the alignment of all available (nearly) full-size trypanosomatid 18S rRNA genes (note that the genera *Trypanosoma* and *Leishmania* were represented by just a subset of the sequenced species) and including three newly obtained sequences (100% identical with the already published sequences of other trypanosomatid isolates) shows the relationship among all known trypanosomatid genera, along with their subfamily affiliations (Fig. 1). The newly erected subfamily Blastocrithidiinae subfam. nov. accommodates the genus *Blastocrithidia* along with the newly establish genus *Obscuromonas* gen. nov. (see below). A close proximity of these two groups was known for a long time (Kostygov and Frolov 2007; Flegontov et al. 2013; Týč et al. 2013; Votýpka et al. 2013; Votýpka et al. 2019, 2020; Králová et al. 2019). In a good correlation with previous studies, in the 18S rRNA-based tree, the '*jaculum*' phylogroup invariably branches in the crown group of the family Trypanosomatidae, with a 100% bootstrap support as a sister clade to the genus *Blastocrithidia* (Fig. 1). Hence, joining these two genera into

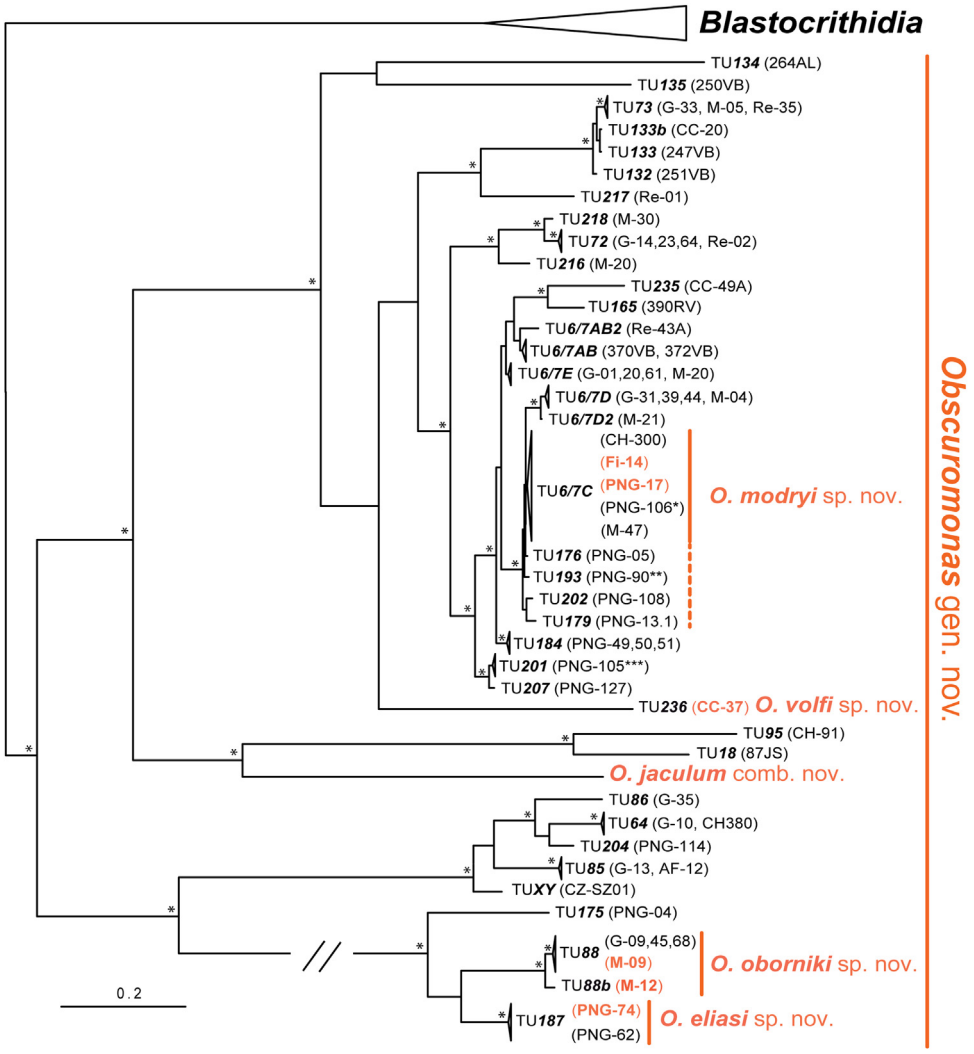

Fig. 2. 18S rRNA-based Bayesian phylogenetic tree of the described species and TUs belonging to the newly established genus *Obscuromonas* gen. nov. Asterisks mark branches with maximal statistical support (bootstrap values for maximum likelihood >90, Bayesian posterior probabilities >0.95); double-crossed branches are 50% of the original length; the scale bar denotes the number of substitutions per site. */**/*** along with the PNG-106/PNG-90/PNG-105 several other DNA isolates from Papua New Guinea belong to the relevant TUs (TU***6/7C***/TU***193***/TU***201***): *PNG-17, 22, 23, 29, 30, 65, 75, 79, 80, 106, 109, 129; **PNG-81, 84.5, 86, 87, 88.2, 89, 90, 91, 93; ***PNG-64, 66, 104, 105, 107, 116, 123.2, 124, 125, 126, 128.2, 130, 131. The geographic origin of the sequenced strains is presented by the prefix as follow: AF – Kenya; CC – Curaçao; CH – China; CZ – Czech Republic; Fi – the Philippines; G – Ghana; M – Madagascar; PNG – Papua New Guinea; Re – Reunion; those starting with a number – South America.

one subfamily is only a logical consequence of the current situation. Along with their phylogenetic affinity, the genera *Blastocrithidia* and *Obscuromonas* gen. nov. share similar host specificity for true bugs, as well as slow growth in culture under a range of conditions, the consequence of which is that out of many known TUs only those described herein are available in culture and, thus, amenable to the morphological and molecular description. Interestingly, members of these two genera share another common feature — their protein-coding genes contain numerous long in-frame insertions, the biological significance of which remains to be elucidated (Nenarokova et al. 2019).

Next, we focused on all the sequences that in the maximum likelihood tree fell into the *Obscuromonas* clade (Fig. 2). The newly obtained 18S rRNA sequence of the cultivable strains Fi-14 from the Philippines and PNG-17 from Papua New Guinea were 100% identical to the several previously published sequences obtained from the DNA of parasites found in the dissected intestine of various heteropteran bugs captured in Papua New Guinea (PNG-17, PNG-22, PNG-23, PNG-29, PNG-30, PNG-65, PNG-76, PNG-79, PNG-80, PNG-106, PNG-109, PNG-129), China (CH-300), and Madagascar (M-47) (Votýpka et al. 2010, 2020; Králová et al. 2019). While they all belong to TU***6/7C*** (Fig. 2), they are closely related to several other TUs from Papua New Guinea, namely TU***176*** (PNG-05), TU***179*** (PNG-13.1), TU***193*** (PNG-90), and TU***202*** (PNG-108), from which they differ by only up to four nucleotides and, thus, may even be considered as

the same species. While future studies will illuminate the prevalence and distribution of the newly described trypanosomatid species, which can vary from the globally distributed *Leptomonas pyrrhocoris* (Votýpka et al. 2012b) to species restricted to tiny populations of their island hosts (Votýpka et al. 2019, 2020), it appears that TU***6/7C*** (*Obscuromonas modryi* sp. nov. (see below)) is widespread, and may even be cosmopolitan.

Two previously published 18S rRNA sequences of the Madagascan cultivable strains M-09 and M-12 belong to TU***88***/TU***88b*** (Votýpka et al. 2020). While the sequence of M-09 is 100% identical with three sequences obtained from parasites infecting the intestine of heteropterans captured in Ghana (Votýpka et al. 2012a), because of the three-nucleotide difference in M12 sequence (100% identity in sequences from the culture and insect host; Votýpka et al. 2020), M-12 has been accommodated into a separate TU, TU***88b*** (Fig. 2). However, based on the above extensive phylogenetic analysis, we propose that both strains represent just different genotypes of the same species.

The newly obtained 18S rRNA sequence of the Papua New Guinean cultivable strain PNG-74 is identical to those of PNG-62 and TU***187***, and closely related to the above-mentioned TU***88***/TU***88b*** (Fig. 2). The last 18S rRNA sequence derived from the Curaçao strain CC-37 (100% identity in sequences from the culture and insect host; Votýpka et al. 2019) represents TU***236*** that is not closely related to any other TUs within the '*jaculum*' clade (Fig. 2).

Morphological and ultrastructural characterization

Examination of all six strains belonging to four different species by light and transmission and scanning electron microscopy revealed a set of features shared by all trypanosomatids, as well as some species-specific traits. The first category is represented by a large nucleus (Figs. 4D, 5 D, 6 H, and 7 G), a single flagellum (Figs. 3A and C, 4 A and C, 5 A and C, 6 A and D, 7 A and D, 8 A–F and H) emerging from a variously extended flagellar pocket (Figs. 3D and G, 4 D, 5 E and D, 6 E, G and H, 7 E and G), a full corset of subpellicular microtubules (Figs. 3F, 6 E), and electron-dense cytoplasmic granules (putative acidocalcisomes) (Figs. 3D and G, 4 D, 5 D and E, 7 G). All trypanosomatids have their flagellum with the canonical 9 + 2 axoneme supported by the paraflagellar rod with a characteristic striated structure, yet its size (Figs. 3E, 5 D and F, 6 E, F and H, 7 F), as well as its length (Figs. 3A, 4 A and C, 5 C and D, 6 A and D, 7 A, D and H) may vary. Indeed, the strains Fi-14 and CC-37 have a prominent rod (Figs. 3E and 5 F), while it is rather small in PNG-74 (Fig. 6F). Moreover, the flagellum cross- and/or longitudinal-sections within the flagellar pocket seems to lack this supportive structure (paraflagellar rod) in PNG-74 (Fig. 6E), yet it is clearly recognizable in M-09, CC-37, and PNG-17 (Figs. 4D, 5 E and 7 E).

The kinetoplast DNA (kDNA) appears as a thin and wide disk in CC-37 (Fig. 5E) and in both strains belonging to TU***6/7C***, Fi-14 (Figs. 3D and G) and PNG-17 (Fig. 4D). In M-12 and PNG-74, the disk is slightly less compact, thicker, and narrow (Figs. 6 G and H, 7 G; Table 2). The fine structural analysis allowed us to note the presence of virus-like particles in Fi-14, where they are enclosed almost exclusively in vesicles (Fig. 3F), while in PNG-74 they are distributed in patches in the cytoplasm; however, some of them are also enclosed in vesicles (Fig. 6G) (Table 2).

Finally, in the strain Fi-14 (Fig. 3F) and both strains belonging to TU***88***, M-09 and M-12, straphangers and cyst-like amastigotes (CLAs) were present in the examined material (Figs. 7C and D, 8 A–Q). Being particularly abundant in the latter strains (M-09 and M-12) (Fig. 8), each pebble-shaped CLA contains in the center of its frontal region a well-visible pinhole, possibly an empty flagellar pocket (Fig. 8H, M, N and O). In sectioned CLAs, we detected a thick cell wall, cytoplasmic membranaceous structures, and numerous granules of irregular electron-density (Fig. 8O–Q). Occasionally, various stages of straphangers/CLAs were observed (Fig. 8I–Q); however, we failed to distinguish any kDNA-like disk or flagellum-like structures in this life cycle stage.

As mentioned above, cultures of *O. modryi* and *O. oborniki* contain the CLAs called also flagellar cysts or straphangers (Fig. 8). The name straphanger reflects the fact that the predecessors of these cyst-like cells are formed *via* unequal division and remain attached for a prolonged period to the flagellum of their mother cell (McGhee and Hanson 1962; Frolov et al. 2018). Straphangers were present in both cultures (M-09 and M-12) of *O. modryi*; in the case of Fi-14 they have been also sporadically observed *in vivo* in the dissected midgut of the host bug (data not shown). Several ultrastructural studies demonstrated that these cyst-like cells are protected by a thick layer of dense submembranous cytoplasm (Frolov and Karpov 1995; Caicedo et al. 2011; Frolov et al. 2018) and are able to efficiently resist the external environment. It was shown that CLAs are present, often in large numbers, in the host rectum, and by the moment of oviposition, they are discharged on the eggs' surface with feces, most likely used for the transmission of bugs' symbiotic bacteria, which are compulsorily engulfed by the newly hatched nymphs along with the CLAs (Frolov et al. 2018).

Although (pre)straphangers observed in culture are transiently attached to the basal part of the flagellum of the mother cell (Figs. 8B–G), in our case this association apparently does not survive the processing for electron microscopy (Figs. 8H–Q). Both the signal that triggers the emergence of straphangers and the sequence that produces from a large standard promastigote/epimastigote cell a shrunk cyst-like straphanger remain obscure, despite their early descriptions and partial insights (Frolov and Karpov 1995; Caicedo et al. 2011; Dias et al. 2014; Frolov et al. 2017, 2018).

Our results are in good correlation with the previous observations and the whole process could be described as the following sequence: mother cell — S1 — S2 — straphanger

Table 2. Morphological analysis of six strains belonging to four newly described species of the genus *Obscuromonas* gen. nov. At least 50 Giemsa-stained cells from cultures were measured and analyzed for their length, width, and flagella length (expressed in micrometers, μm). Measurements for kinetoplast thickness and wideness were performed for at least 30 cultivated cells and virus diameters were calculated by analyzing at least 25 particles (TEM, expressed in nanometers, nm). Values are expressed as range MIN–MAX (mean ± SE); CLAs – cyst-like amastigotes.

	O. modryi		*O. oborniki*				*O. volfi*	*O. eliasi*
	Fi-14	PNG-17	M-09		M-12		CC-37	PNG-74
	Promastigotes	Promastigotes	Promastigotes	CLAs	Promastigotes	CLAs	Promastigotes	Promastigotes
Cell length	13.0–26.7 (18.0 ± 3.3)	9.5–24.6 (16.3 ± 3.1)	15.9–35.0 (21.3 ± 4.6)	2.4–3.4 (2.9 ± 0.2)	13.0–26.4 (18.0 ± 2.8)	1.6–4.2 (3.1 ± 0.4)	7.9–17.8 (12.2 ± 2.5)	11.5–25.3 (15.0 ± 2.4)
Cell width	2.2–4.5 (2.7 ± 0.5)	1.6–3.3 (2.1 ± 0.3)	1.6–3.3 (2.3 ± 0.4)	1.1–1.8 (1.4 ± 0.1)	1.4–2.7 (1.9 ± 0.3)	1.1–3.2 (1.6 ± 0.3)	3.0–7.2 (4.6 ± 0.8)	1.7–3.4 (2.4 ± 0.4)
Flagella length	4.9–23.8 (14.0 ± 5.1)	7.1–29.2 (18.7 ± 4.0)	24.4–41.3 (32.7 ± 5.0)	N/A	16.1–41.7 (32.1 ± 5.6)	N/A	6.9–28.7 (13.7 ± 4.9)	12.2–35.6 (23.5 ± 5.2)
Kinetoplast thickness	103.9–204.5 (141.6 ± 26.5)	116.3–162.1 (131.3 ± 14.0)	189.5–267.7 (216.8 ± 19.9)	N/A	N/A	N/A	88.5–130.9 (106.5 ± 10.3)	198.0–294.8 (231.5 ± 21.5)
Kinetoplast wideness	610.5–1450.2 (1036.6 ± 208.3)	585.8–1372.1 (947.2 ± 234.9)	501.7–784.3 (627.2 ± 75.4)	N/A	N/A	N/A	801.5–1430.7 (949.5 ± 168.1)	465.5–1025.6 (825.3 ± 139.1)
Virus diameter	27.1–44.3 (35.4 ± 4.3)	N/A	N/A	N/A	N/A	N/A	N/A	45.5–54.5 (49.1 ± 3.4)

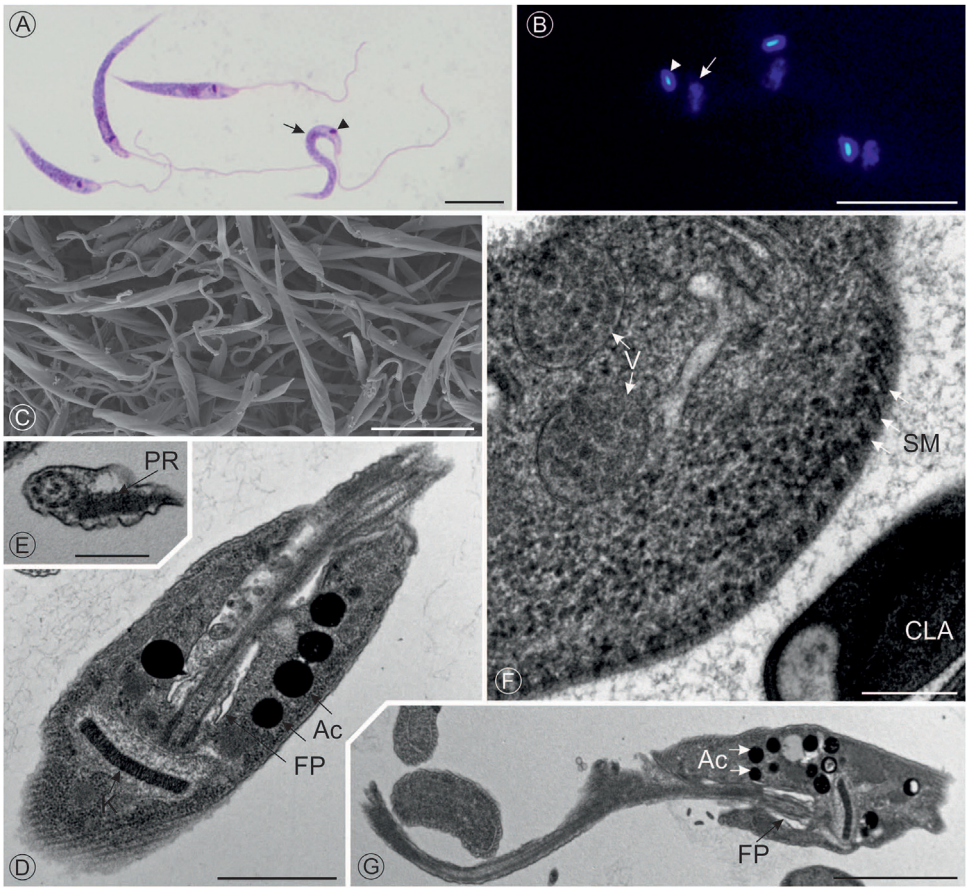

Fig. 3. ***Obscuromonas modryi*** sp. nov. (strain Fi-14). Giemsa-stained (**A**) and DAPI-stained cultured cells (**B**). The nucleus and the kinetoplast DNA are labeled with arrow and arrowhead, respectively. (**C**) Scanning electron micrograph of dense promastigote culture. (**D**) Section thru the flagellar pocket containing numerous dense acidocalcisomes (Ac), and thin and wide kinetoplast DNA disk (K). (**E**) The cross-sectioned external flagellum is equipped with a prominent paraflagellar rod. (**F**) Two cross-sectioned cytoplasmic vesicles containing virus-like particles (V). Note the regularly spaced subpellicular microtubules (SM) and cyst-like amastigotes (CLA). (**G**) Longitudinal section thru a rather deep flagellar pocket (FP) surrounded by cytoplasm rich in acidocalcisomes (Ac). Scale bars: A–C = 10 um; G = 2 um; E = 1 um; F = 200 nm; D = 500 nm.

— CLA. After an unequal division, the daughter cell (S1) does not grow the long flagellum and stays attached to the flagellum of the mother cell (Figs. 8A and D; 8 I and J show the S1 cells artificially disconnected from their mother cells). While in *Blastocrithidia papi* the CLAs are formed from epimastigotes (Frolov et al. 2018), in *Obscuromonas* spp. promastigotes constitute the mother cell. Following fission of the S1 cell, the size of which is comparable with that of the mother cell, two (likely) non-dividing S2 cells, which are noticeably smaller (Fig. 8B, C, and E), are formed. These twins eventually lose their connection to the maternal flagellum, yet stay attached to its similarly-sized partner (Fig. 8L). Next, the S2 cell starts progressively shrinking until it reaches the pebble-shaped form of straphangers (Fig. 8C–F, K), still being attached to the maternal flagellum. The next division of the mother cell, which retains the promastigote morphology, again results in the production of another S1 cell (Fig. 8D), and the whole S1 – S2 – straphanger sequence may be repeated several times (Fig. 8C–F). The newly formed straphangers either remain attached to the mother flagellate, along with straphangers that are products of the previous division, or they detach, transform into the CLAs, and remains free in the lumen of the intestine (or at the bottom of the culture flask). With the aging of the culture, the proportion of free CLAs progressively grows, until they represent the most frequent morphotype (Figs. 7D and 8 H). The S1 (Fig. 8I and J), S2 (Fig. 8L), and early straphangers (Fig. 8H and K) still contain a rudimentary flagellum, which mediates the connection to the mother/sister cell. We speculate that following the detachment from the mother cell, straphanger discards its own flagellum and transforms into a mature CLAs with an empty flagellar pocket (Figs. 7D and 8 H, M, N, and O). Curiously, while we did not see the disk-shaped densely packed kDNA in the fine structure of the cross-sectioned CLAs by TEM (Figs. 8O–Q), in light microscopy the Giemsa staining and DAPI-labeling clearly revealed its presence (Fig. 8B–G). Indeed, the characteristic electron-dense kDNA disk is not visible even in the fine structure of straphangers/CLAs presented in previous studies (Caicedo et al. 2011; Frolov et al. 2017, 2018). One of the

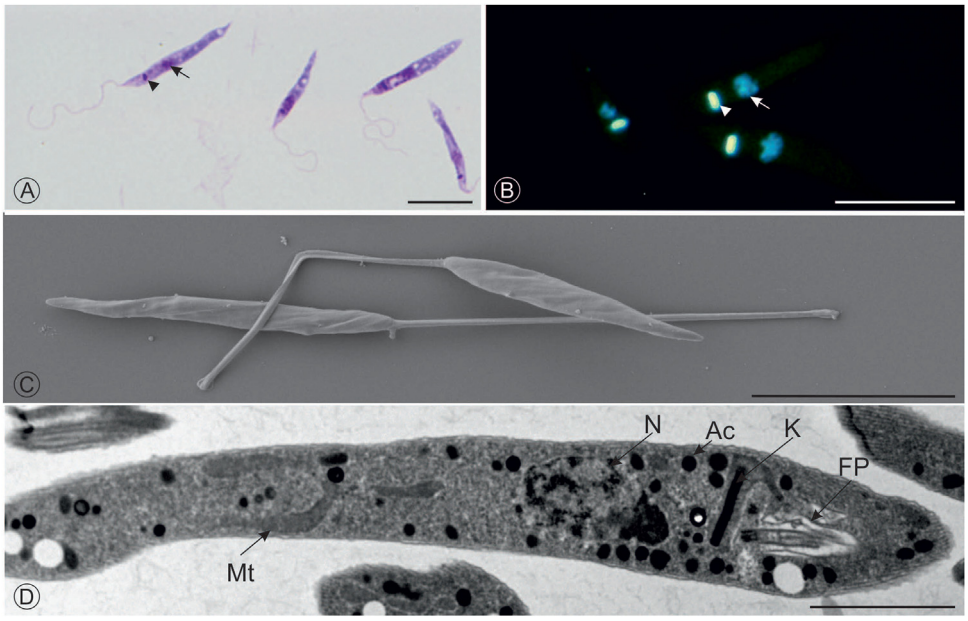

Fig. 4. ***Obscuromonas modryi*** sp. nov. (strain PNG-17). Giemsa-stained (**A**) and DAPI-stained cultured cells (**B**). The nucleus and the kinetoplast DNA are labeled with arrow and arrowhead, respectively. (**C**) Scanning electron micrograph of typical promastigotes. (**D**) Longitudinal section showing the anterior position of the nucleus (N), reticulated mitochondrion (Mt), a very thin kinetoplast DNA disk (K), and numerous acidocalcisomes (Ac). Scale bars: A–C = 10 um; D = 2 um.

possible explanations of this phenomenon is that the kDNA network may disintegrate in CLAs.

Another interesting trait worth noting is the stable and prominent presence of viruses in the cytoplasm of *O. modryi* (strain Fi-14) and *O. eliasi* (Figs. 3F and 6 G; Table 2). Our observation that only one (Fi-14) out of two (Fi-14 and PNG-17) studied strains belonging to the same species (*O. modryi*) is infected with viruses confirms a previous finding that the presence of viruses is not species- but rather strain-specific (Grybchuk et al. 2018a). Although monoxenous trypanosomatids were recently shown to host a large diversity of viruses (Grybchuk et al. 2018a,b), in the absence of molecular data the appurtenance of the viruses observed in *Obscuromonas* remains unknown.

In this work, we have formally described a long-known, geographically widespread clade of trypanosomatids that infects a wide range of heteropteran hosts. It resisted a proper ranking and in-depth studies since its representatives could not have been axenically cultivated. That has now changed, and the available stabilates are accessible for future studies.

Taxonomic summary

Class Kinetoplastea Honigberg, 1963 emend. Vickerman, 1976

Subclass Metakinetoplastina Vickerman, 2004

Order Trypanosomatida Kent, 1880

Family Trypanosomatidae Doflein, 1901

Subfamily Blastocrithidiinae Votýpka, Yurchenko et Lukeš, subfam. nov.

Type genus. *Blastocrithidia* Laird, 1959, here designated (type species: *Crithidia gerridis* Patton, 1908).

Diagnosis. A well-supported monophyletic group of monoxenous trypanosomatid parasites in the gut of true bugs (Heteroptera). (Note: The forming of straphangers (cyst-like amastigotes) is typical for some species.)

Included genera. *Blastocrithidia* Laird, 1959 and *Obscuromonas* gen. nov.

Etymology: The name of the subfamily has originated from the name of the first described genus of this clade, *Blastocrithidia*.

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Genus *Obscuromonas* Votýpka et Lukeš 2020, gen. nov.

Type species. *Obscuromonas modryi* Votýpka et Lukeš, sp. nov., here designated.

Diagnosis. A well-supported monophyletic group of monoxenous trypanosomatids parasitic in the gut of true bugs (Heteroptera). It is defined by a set of unique sequences of the 18S rRNA gene and its position on the 18S rRNA-based

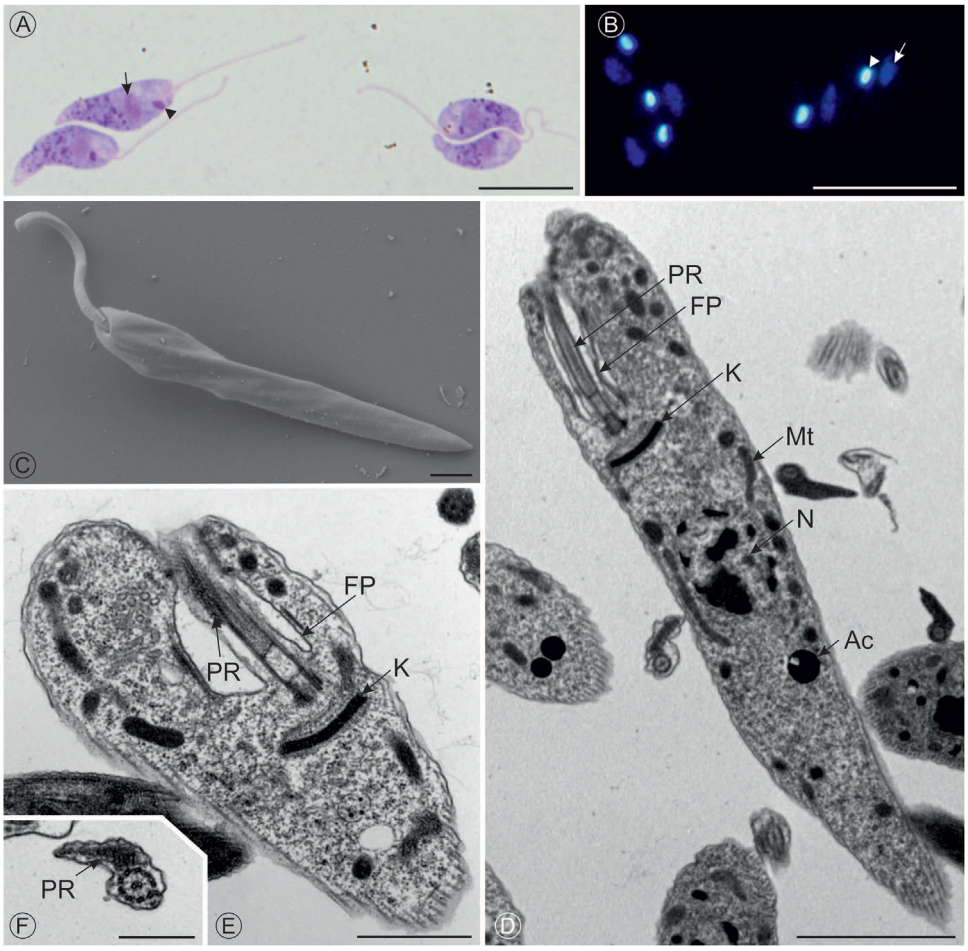

Fig. 5. ***Obscuromonas volfi*** sp. nov. (CC-37). Giemsa-stained (**A**) and DAPI-stained cultured cells (**B**). The nucleus and the kinetoplast DNA are labeled with arrow and arrowhead, respectively. (**C**) Scanning electron micrograph of a slender-shaped promastigote. (**D**) Longitudinal section thru the promastigote, showing central nucleus (N), peripheral mitochondrion (Mt), putative acidocalcisomes (Ac), and kinetoplast DNA disk (K). (**E**) A very thin kinetoplast DNA disk (K) is located at the base of a relatively narrow flagellar pocket. (**F**) The cross-sectioned external flagellum is equipped with a prominent paraflagellar rod (PR). Scale bars: A and B = 10 um; D = 2 um; C and E = 1 um; F = 500 nm.

phylogenetic tree. Molecular phylogenetic analyses confirm this monophyletic genus as a member of the family Trypanosomatidae closely related to the genus *Blastocrithidia*, but is distinct from it. The type species of the genus is *Obscuromonas modryi* sp. nov.

Etymology. The generic name refers to an unusual situation where it has long been known that there is a monophyletic, species-rich and widely geographically distributed clade of monoxenous trypanosomatids infecting a wide range of heteropteran host species, but because available strains could not be cultivated, it was not possible to describe these parasites in sufficient detail and, thus, formally establish a new genus. Latin name *obscuro* means “cause to be forgotten”; Greek *monas* (monad, third declension, monas, feminine) is included in many generic names of flagellates.

The ZooBank LSID (Life Science Identifier): urn:lsid:zoobank.org:pub: 43FCE707-15A8-47BC-908C-36F5A82408B8.

Obscuromonas modryi Votýpka et Lukeš, sp. nov.

Diagnosis. The species is identified by the unique 18S rRNA sequence with GenBank acc. number MW177946.

Morphological description (Fig. 3, Tables 1 and 2). The culture is dominated by promastigotes; *in vivo* promastigotes were observed with sporadic straphangers. Cells in culture range from 13.0 to 26.7 (18.0 ± 3.3) μm long and from 2.2 to 4.5 (2.7 ± 0.5) μm wide with flagella varying from 4.9 to 23.8 (14.0 ± 5.1) μm. The kDNA disk is compactly packed and its thickness varies between 103.9 and 204.5 (141.6 ± 26.5) nm and width is between 610.5 and 1450.2 (1036.6 ± 208.3) nm. The presence of cytoplasmic viruses is notable, but not a species diagnostic feature.

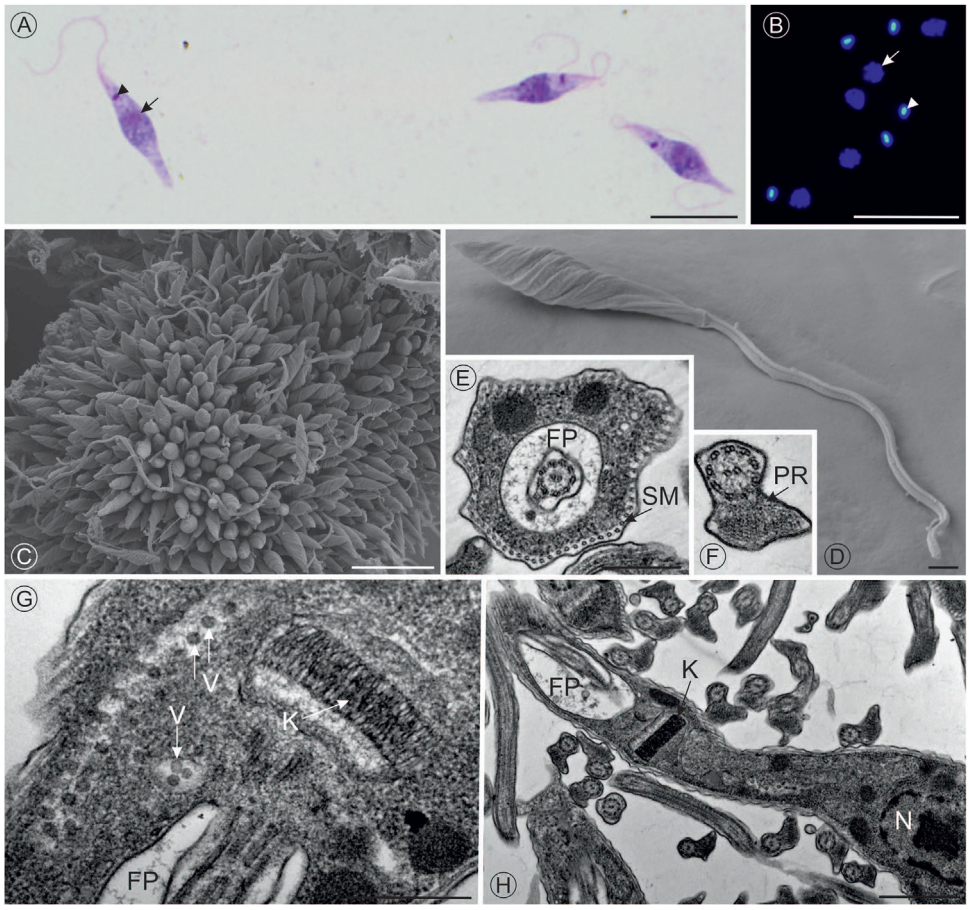

Fig. 6. ***Obscuromonas eliasi*** sp. nov. (strain PNG-74). Giemsa-stained (**A**) and DAPI-stained cultured cells (**B**). The nucleus and the kinetoplast DNA are labeled with arrow and arrowhead, respectively. Scanning electron micrograph of cultured cells forming large rosettes (**C**) and screw-shaped promastigote (**D**). Within the flagellar pocket (FP), the flagellum lacks the paraflagellar rod (**E**), which appears in a rather prominent form (PR) in the free part of the flagellum (**F**). (**G**) Small clumps of viruses (V) are found in the periflagellar region of the cytoplasm. (**H**) Longitudinal section revealing the central nucleus (N), prominent flagellar pocket (FP), and thick and rather narrow kinetoplast DNA disk (K). Scale bars: A–C = 10 um; D and H = 1 um; E and G = 500 nm; F = 200 nm.

Type host and site. The midgut of *Riptortus linearis* (Fabricius, 1775) (Alydidae: Alydinae).

Type locality. The Philippines, Luzon, Bontoc (17°5′58″N, 120°59′22″E).

Type material. The name-bearing type, a hapantotype, is a Giemsa-stained slide of the primary strain **Fi-14**, deposited in the collection of the Department of Parasitology, Faculty of Science, Charles University, Prague (acc. code: 2013/Fi-14/S). Axenic culture of the primary isolate Fi-14 is deposited in the above collection and in the Institute of Parasitology, České Budějovice, Czech Republic (acc. code: Fi-14).

Etymology. The species name *modryi* is dedicated to David Modrý, University of Veterinary and Pharmaceutical Sciences, for his major contribution to veterinary parasitology.

Remarks. An identical 18S rRNA sequence was obtained from the strain PNG-17 (MW177948), which has been isolated from *Riptortus linearis* (Alydidae) in Papua New Guinea and is here also described in some detail (Fig. 4, Tables 1 and 2). Based on this sequence identity, we conclude that several other true bug species serve as hosts of *Obscuromonas modryi*: *Leptocorica* (Alydidae: Micrelytrinae) and *Riptortus* spp. from Papua New Guinea, China, and Madagascar, and *Hydara* (Coreidae: Hydarinae) and *Clavigralla* spp. (Coreidae: Pseudophloeinae: Clavigrallini) from Madagascar (Votýpka et al. 2010, 2012a, 2020; Králová et al. 2019).

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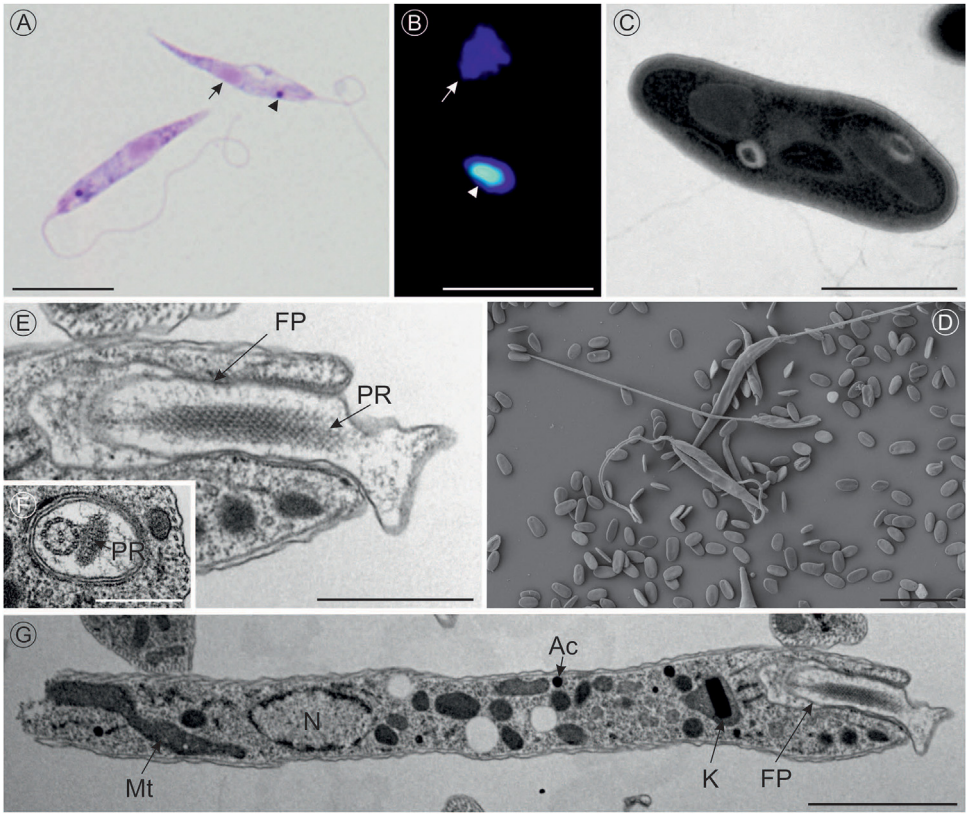

Fig. 7. ***Obscuromonas oborniki*** sp. nov. (strain M-09). Giemsa-stained (**A**) and DAPI-stained cultured cells (**B**). The nucleus and the kinetoplast DNA are labeled with arrow and arrowhead, respectively. (**C**) Longitudinal section of a mature cyst-like amastigote (CLA). (**D**) Scanning electron micrograph of cultured promastigotes largely outnumbered by CLAs. Longitudinal (**E**) and cross-section (**F**) thru the flagellum within the flagellar pocket, revealing the paraflagellar rod (PR) already within the flagellar pocket. (**G**) Longitudinally sectioned slender promastigote, revealing a rather dorsal position of the nucleus (N) and thick and narrow kinetoplast DNA disk (K). Scale bars: A and D = 10 um; B = 5 um; G = 2 um; C and E = 1 um; F = 500 nm.

Obscuromonas volfi Votýpka et Lukeš, sp. nov.

Diagnosis. The species is identified by the unique 18S rRNA sequence with GenBank acc. number MK056199.

Morphological description (Fig. 5, Tables 1 and 2). The culture is dominated by promastigotes, which were also observed *in vivo*. Cells in culture range from 7.9 to 17.8 (12.2 ± 2.5) μm long and from 3.0 to 7.2 (4.6 ± 0.8) μm wide with flagella varying from 6.9 to 28.7 (13.7 ± 4.9) μm. The kDNA disk is compactly packed and its thickness varies between 88.5 and 130.9 (106.5 ± 10.3) nm and width ranges between 801.5 and 1430.7 (949.5 ± 168.1) nm.

Type host and site. The midgut and hindgut of *Catorhintha selector* Stål, 1860 (Coreidae: Coreinae: Hypselonotini).

Type locality. Caribbean island of Curaçao, Souax (12°10′45.09″N, 68°57′54.28″W).

Type material. The name-bearing type, a hapantotype, is a Giemsa-stained slide of the primary isolate **CC-37**, deposited in the collection of the Department of Parasitology, Faculty of Science, Charles University, Prague (acc. code: 2015/CC-37/S). Axenic culture of the primary isolate CC-37 is deposited in the above collection and in the Institute of Parasitology, České Budějovice, Czech Republic (acc. code: CC-37A).

Etymology. The species name *volfi* is dedicated to Petr Volf, Charles University, for his major contribution to our understanding of the relationship between trypanosomatids and the phlebotomine flies.

Remarks. Based on the identical 18S rRNA gene sequences, another member of the genus *Catorhintha* (Coreidae), *C. guttula*, and *Neomegalotomus parvus* (Alydidae: Alydinae) may serve as hosts of *Obscuromonas volfi* in Curaçao (Votýpka et al. 2019).

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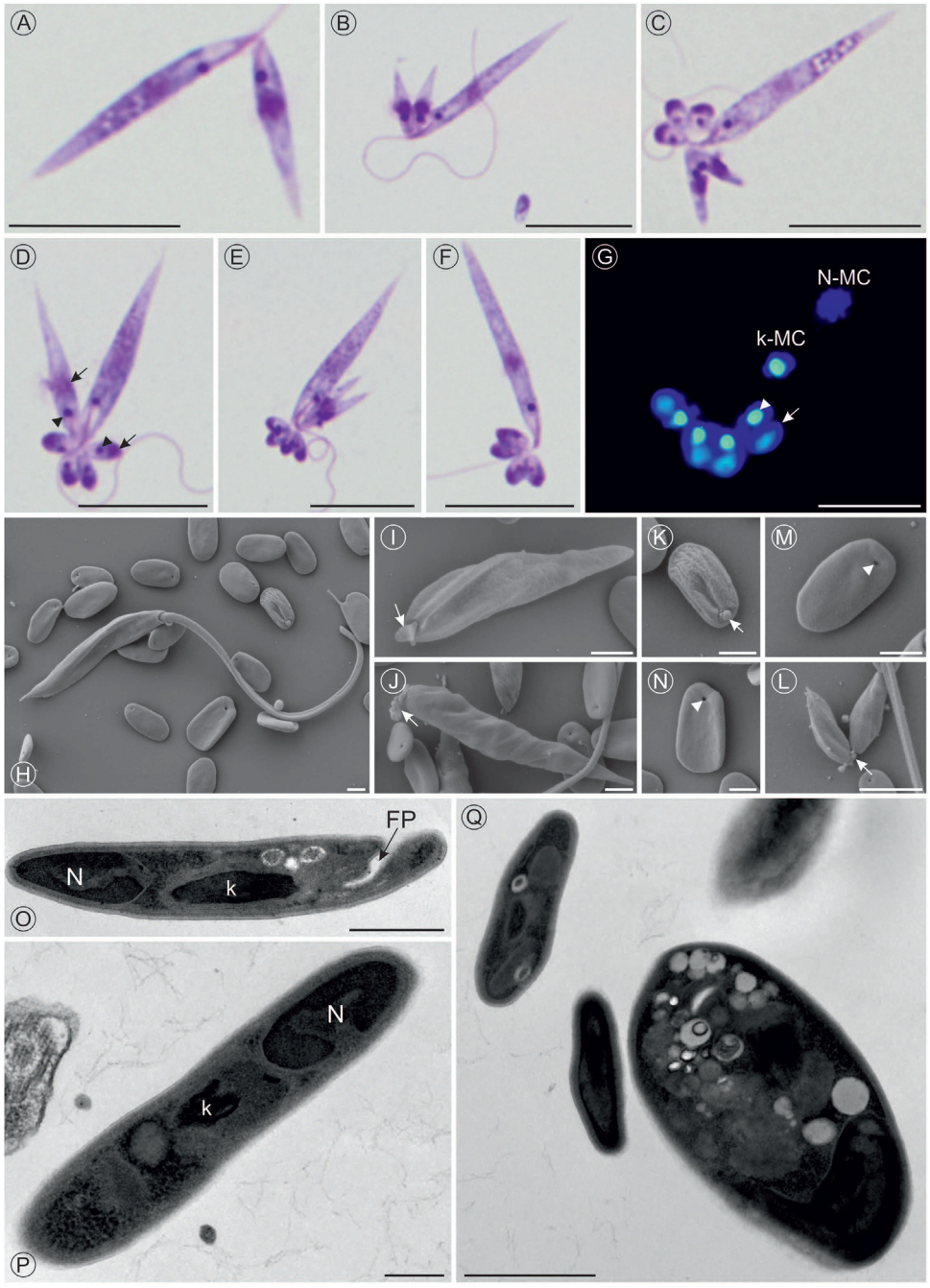

Fig. 8. ***Obscuromonas oborniki*** sp. nov. (strain M-12). Giemsa-stained (**A** thru **F**) cultured cells with different stages of the straphanger formation. (**A**) Promastigote mother cell with attached daughter cell (S1). (**B**) Promastigote mother cell with an attached twin of S2 cells, similar in size, which originates from S1 cell. (**C**) Promastigote mother cell with the twin of S2 cells and three mature straphangers (arise from S2 cells). (**D**) Promastigote mother cell with one daughter cell (S1) and two twins of mature straphangers. (**E**) Promastigote mother cell with one twin of S2 cells and two twins of mature straphangers. (**F**) Promastigote with two twins of mature straphangers. Note that the flagellum is invariably present only in the mother cells. (**G**) DAPI-stained promastigote mother cell (MC) with four mature straphangers. Note that both Giemsa and DAPI visualize the distinct kinetoplast DNA and nucleus of the promastigote mother cell (k-MC and N-MC), as well as the kinetoplast DNA (arrowhead) and nucleus (arrow) of the straphangers. (**H**). Representative photo of a dense culture, in which free cyst-like amastigotes (CLAs) are much more abundant than the flagellates. (**I** and **J**) S1 cells with the rudimentary flagellum (arrow) artificially separated from their mother cells. (**K**) Straphanger and (**L**) twin of S2 cells with the rudimentary flagellum (arrow) artificially separated from their mother cells. (**M** and **N**) Mature cyst-like amastigotes (CLAs) with an empty flagellar pocket (arrowhead). (**O**) Longitudinal sections thru a mature CLA with cross-sectioned rudimentary flagellar pocket (FP), kinetoplast DNA (k), and nucleus (N). (**P**) Longitudinal section thru a mature CLA. (**Q**) Cross-sectioned CLAs at various stages of their maturation. Scale bars: A–G = 10 um; L = 5 um; H–J, Q = 1 um; K, M, N and O = 500 nm; P = 200 nm.

Obscuromonas eliasi Votýpka et Lukeš, sp. nov.

Diagnosis. The species is identified by the unique 18S rRNA sequence with GenBank acc. number MW177947.

Morphological description (Fig. 6, Tables 1 and 2). The culture is dominated by promastigotes that were *in vivo* observed exclusively in Malpighian tubules. Cells in culture range from 11.5 to 25.3 (15.0 ± 2.4) μm long and from 1.7 to 3.4 (2.4 ± 0.4) μm wide with flagella varying from 12.2 to 35.6 (23.5 ± 5.2) μm. The kDNA disk is compactly packed and its thickness varies between 198.0 and 294.8 (231.5 ± 21.5) nm, width between 465.5 and 1025.6 (825.3 ± 139.1) nm. Notable is the presence of viruses freely in the cytoplasm.

Type host and site. The Malpighian tubes of *Graptostethus servus* (Fabricius, 1787) (Lygaeidae: Lygaeinae).

Type locality. Papua New Guinea, Nagada (5°9′23″S, 145°47′41″E)

Type material. The name-bearing type, a hapantotype, is a Giemsa-stained slide of the primary isolate **PNG-74**, deposited in the collection of the Department of Parasitology, Faculty of Science, Charles University, Prague (acc. code: 2011/PNG-74/S). Axenic culture of the primary isolate PNG-74 is deposited in the research collections of the Department of Parasitology, Faculty of Science, Charles University, Prague and in the Institute of Parasitology, České Budějovice, Czech Republic (acc. code: PNG-74).

Etymology. The species name *eliasi* is dedicated to Marek Eliáš, University of Ostrava, for his significant contributions to our understanding of the evolution and genomics of parasitic protists.

Remarks. Based on the identical sequence of the 18S rRNA gene, we propose that the assassin bug *Helonotus* sp. (Reduviidae: Harpactorinae) in Papua New Guinea (Králová et al. 2019) may also serve as a host of *Obscuromonas eliasi*. However, due to a very low intensity of the infection, it is also possible that this predatory bug acquired the flagellate from its prey.

The ZooBank LSID (Life Science Identifier): urn:lsid:zoobank.org:pub: 43FCE707-15A8-47BC-908C-36F5A82408B8.

Obscuromonas oborniki Votýpka et Lukeš, sp. nov.

Diagnosis. The species is identified by the unique 18S rRNA sequence with GenBank acc. number MT174475.

Morphological description (Fig. 7, Tables 1 and 2). In culture, promastigotes and/or promastigotes with straphangers are dominating forms from the beginning, later on, cyst-like amastigotes are more prevalent; *in vivo* long promastigotes were observed. Promastigotes in culture range from 15.9 to 35.0 (21.3 ± 4.6) μm long and from 1.6 to 3.3 (2.3 ± 0.4) μm wide with flagella varying from 24.4 to 41.3 (32.7 ± 5.0) μm; the kinetoplast disk is compactly packed and its thickness varies between 189.5 and 267.7 (216.8 ± 19.9) nm and width ranges from 501.7 to 784.3 (627.2 ± 75.4) nm. Cyst-like amastigotes in culture range from 2.4 to 3.4 (2.9 ± 0.2) μm long and from 1.1 to 1.8 (1.4 ± 0.1) μm wide.

Type host and site. The midgut of *Aspilocoryphus unimaculatus* (Signoret, 1860) (Lygaeidae: Lygaeinae).

Type locality. Madagascar, Ambatofosty (19°16′46.79″S, 47°28′44.47″E).

Type material. The name-bearing type, a hapantotype, is a Giemsa-stained slide of the primary strain **M-09**, deposited in the collection of the Department of Parasitology, Faculty of Science, Charles University, Prague (acc. code: 2010/M-09/S). Axenic culture of the primary isolate M-09 is deposited in the above collection and in the Institute of Parasitology, České Budějovice, Czech Republic (acc. code: M-09).

Etymology. The species name *oborniki* is dedicated to Miroslav Oborník, Biology Centre of the Czech Academy of Sciences, who made a significant contribution to our knowledge about the phylogeny of parasitic protists.

Remarks. An almost identical 18S rRNA sequence was obtained from the strain M-12 isolated from *Spilostethus pandurus* (Lygaeidae: Lygaeinae) captured in Madagascar, described in some detail in this study (Fig. 8, Tables 1 and 2). Based on this sequence identity, we conclude that another true bug species from Ghana, *Aspilocoryphus fasciaventris* (the same genus as the type host), serves as a host of *Obscuromonas oborniki* (Votýpka et al. 2012a).

The ZooBank LSID (Life Science Identifier): urn:lsid:zoobank.org:pub: 43FCE707-15A8-47BC-908C-36F5A82408B8.

Obscuromonas jaculum (Léger, 1902) comb. nov.

This species corresponds to former *Herpetomonas jaculum* Léger, 1902 detected in the intestine of the true bug *Nepa cinerea* (water scorpion, Nepidae) collected in France more than 100 years ago (Léger 1902a, b). In 1916, the species was transferred to another genus (Woodcock 1916) and renamed *Leptomonas jaculum* (Léger, 1902) Woodcock, 1916.

Note: The species has never been recovered in culture. However, sequence analysis of parasites of the same morphotype as in the original description and isolated from the same host species revealed the affinity of *Leptomonas jaculum* to the genus *Blastocrithidia* (Kostygov and Frolov, 2007). Since this species was for a long time the only known representa-

tive of this clade, the monophyletic clade was provisionally labeled '*jaculum*'.

The ZooBank LSID (Life Science Identifier): urn:lsid:zoobank.org:pub: 43FCE707-15A8-47BC-908C-36F5A82408B8.

CRediT authorship contribution statement

Julius Lukeš: Conceptualization, Writing - review & editing. **Martina Tesařová:** Visualization. **Vyacheslav Yurchenko:** Writing - review & editing. **Jan Votýpka:** Conceptualization, Methodology, Writing - original draft, Writing - review & editing.

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