J. Parasitol., 90(2), 2004, pp. 342–347

HERPETOMONAS ZTIPLIKA N. SP. (KINETOPLASTIDA: TRYPANOSOMATIDAE): A PARASITE OF THE BLOOD-SUCKING BITING MIDGE *CULICOIDES KIBUNENSIS* TOKUNAGA, 1937 (DIPTERA: CERATOPOGONIDAE)

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ABSTRACT: Herein, we describe the first case of a natural infection of biting midges by a kinetoplastid protozoan. Flagellates from a female *Culicoides kibunensis* captured in a bird's nest were introduced into culture and characterized by light and electron microscopy. However, because the morphological data were inconclusive, the novel endosymbiont-free trypanosomatid was assigned into *Herpetomonas* primarily on the basis of the 18S and 5S ribosomal RNA (rRNA) gene sequences.

Kinetoplastid flagellates belonging to the Trypanosomatidae are frequently found as parasites of insects and are especially abundant in members of 2 orders, Diptera and Hemiptera. Tsetse flies (*Glossina* spp.), sand flies (*Phlebotomus* spp. and *Lutzomyia* spp.), and triatomine bugs (*Triatoma* spp., *Rhodnius* spp., and *Panstrongylus* spp.) serve as vectors that transmit flagellate species of *Trypanosoma* and *Leishmania*, causative agents of many serious diseases in vertebrate hosts, including humans. Insects also host less well-known species of monoxenic trypanosomatids of *Leptomonas*, *Crithidia*, *Blastocrithidia*, *Wallaceina*, *Rhynchoidomonas*, and *Herpetomonas* (Wallace, 1966; Podlipaev, 1990). Taxonomy of these genera remains elusive because of the lack of clear-cut morphological criteria (Nunes et al., 1994; Podlipaev, 2000).

Interestingly, the vectors of trypanosomes and leishmanias appear to be almost free of monoxenic trypanosomatids. *Blastocrithidia triatomae* from the triatomine bugs (Cerisola et al., 1971) that transmit *Trypanosoma cruzi*, responsible for Chagas disease, represents the only well-documented exception. Moreover, a handful of unconfirmed reports of the tsetse and sand flies infected with *Leptomonas* and *Crithidia* exist in the literature (Podlipaev, 1990). Considering the century-long intense investigation of the aforementioned insects as vectors of trypanosomes and leishmanias, such a low number of reports on their monoxenic trypanosomatids may reflect either a real absence of these species or a general negligence toward medically unimportant flagellates.

Insect flagellates appear to have a low level of host specificity and high physiological variability, which may facilitate establishment of a new host–parasite system (Podlipaev, 2000). The presence of monoxenic trypanosomatids in insects actively attacking humans requires particular attention because, under favorable conditions, these parasites may develop infections in immunocompromised patients. It is possible that recent reports regarding monoxenous-like trypanosomatids infecting humans (Dedet and Pratlong, 2000; Noyes et al., 2002) fall into this category.

Herein, we describe a new *Herpetomonas* species from a biting midge, *Culicoides kibunensis* Tokunaga, 1937 (Diptera: Ceratopogonidae). In large areas of the Northern Hemisphere, these tiny dipterans feed voraciously on the blood of warm-blooded vertebrates and are second only to mosquitoes as pests of humans and livestock. They attack humans, producing in some cases a significant economic impact on tourism and outdoor activities, and may also cause serious dermatitis and allergic reaction in sensitive individuals. At the same time, biting midges are well-known vectors of animal diseases, such as bird and lizard malaria, equine onchocerciasis, blue tongue virus of wild and domestic ruminants, and the oropouche virus infecting humans. Moreover, it was experimentally demonstrated that several *Culicoides* species serve as vectors of avian trypanosomes (Miltgen and Landau, 1982). Although these experimental infections have not been confirmed (Votýpka et al., 2002), midges from the species-rich Ceratopogonidae appear so far to be free of trypanosomatids.

The present description of a new trypanosomatid species, which to our knowledge is the first from biting midges, is based on morphological considerations, as well as on the sequences of the small subunit (18S) and 5S rRNA genes. Phylogenetic analysis of the sequence data led us to assign the flagellate in question to *Herpetomonas*.

MATERIALS AND METHODS

Organism

Herpetomonas ztiplika n. sp. was isolated from a female *Culicoides* (*Oecacta*) *kibunensis* Tokunaga, 1937 (synonym *C. cubitalis* Edwards, 1939) caught while attacking buzzard (*Buteo buteo*) nestlings in an oak–hornbeam forest in the vicinity of Mikulov, southern Moravia, Czech Republic (48°50′N, 16°41′E). Insects were collected using air-sucking miniature Centers for Disease Control traps without a light bulb placed at the level of a bird's nest, 16 m above the ground. Trapped midges were dissected under a stereomicroscope, and their alimentary tracts were examined using light microscopy. The contents of the alimentary tract were inoculated into rabbit blood agar (SNB-9) supplemented with gentamicin (80 μg/ml). The culture was kept at 23 C, passaged weekly, and stored in the cryobank of the Department of Parasitology, Faculty of Science, Charles University, Prague, as strain ICUL/CZ/1999/CER1.

DNA extraction, polymerase chain reaction, and sequencing

Isolation of total cell DNA and kinetoplast DNA (kDNA) network from *H. ztiplika* n. sp., polymerase chain reaction amplification, cloning, and sequencing of the 18S rRNA gene have been described elsewhere (Votýpka et al., 2002). The 5S rRNA gene repeat was amplified using oligonucleotides and conditions of Dollet et al. (2000). Amplicon was purified on a 0.75% agarose gel, gel isolated, and cloned using TOPO™ TA cloning kit version E (Invitrogen, Carlsbad, California). Both strands were sequenced on an automated DNA sequencer using the CEQ™ DTCS kit (Beckman, Fullerton, California).

Received 27 May 2003; revised 15 August 2003; accepted 18 August 2003.
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Light and electron microscopy

For light microscopy, smeared cells from a 6-day-old culture were fixed for 5 min with methanol and stained with Giemsa (Sigma, St. Louis, Missouri) for 30 min, or incubated for 3 min in phosphate-buffered saline with 0.1 μg/ml 4′,6-diamidine-2-phenylindole (DAPI) at room temperature, and examined with an Olympus BX60 microscope. Fifty cells were measured with a calibrated micrometer. Cultivation was also done at elevated temperatures (30–33 C) to induce possible transformation into opistomastigotes.

For transmission electron microscopy, cells collected from a 6-day-old culture were washed in 0.1 M phosphate-buffered saline solution and fixed in 2.5% glutaraldehyde in the same buffer for 1 hr at 4 C. After dehydration in graded series of ethanol, the cells were embedded in Epon-Araldite, thin sections were stained with lead citrate and uranyl acetate, and they were examined under a JEOL 1010 microscope.

Cultured cells were spotted onto poly-L-lysine–coated glass cover slips and fixed in 2% OsO_4 in 0.2 M cacodylate buffer at 4 C for 1 hr and washed in the same buffer at 4 C for 1 hr. They were dehydrated, infiltrated with increasing concentrations of acetate, critical-point air dried, sputter coated with gold in a Polaron coater, and examined using a JEOL 6300 scanning electron microscope.

Phylogenetic analysis

Phylogenetic analysis was performed using maximum parsimony (MP), maximum likelihood (ML), and distance (minimum evolution) (ME) methods, carried out using the program package PAUP*, version 4.0b10 (Swofford, 2001). The MP analysis was done using heuristic search with random addition of taxa (10 replications) and the ACCTRAN option. Gaps were treated as missing data. Four different transversion–transition (Tv–Ts) ratios were tested (1:2 to 1:5). To obtain the best tree using the ML analysis, the likelihood ratio test implemented in Modeltest ver. 3.06 (Posada and Crandall, 1998) was used, in which the GTR + Γ + G model of evolution was found to fit the data best. The distance method was executed using heuristic search with ME as the objective setting and the K2P substitution model. Clade support was assessed with bootstrapping (1,000 replicates for MP and ME, 500 replicates for ML).

The following 18S and 5S sequences retrieved from the GenBank were included in the analysis: *Wallaceina inconstans* (AF153044), *Crithidia fasciculata* (Y00055), *Leptomonas seymouri* (AF153040), *Leishmania donovani* (X07773), *Endotrypanum monterogei* (X543911), *H. megaseliae* (U01014), *H. muscarum* (L18872), *H. pessoai* (U01016), *H. mariadeanei* (U01013), *Herpetomonas* sp. TCC263 (AF038024), *H.* cf. *roitmani* (AF267738), *H. roitmani* (AF038023), *Herpetomonas* sp. CER1 (*H. ztiplika* sp. n.) (AF416560), *B. culicis* (U05659), *Crithidia oncopelti* (AF038025), *T. avium* (U39578), *T. cruzi* (M31432), *Bodo saltans* (AF208887), *Trypanoplasma borreli* (L14840), *Phytomonas* sp. E.hi. Se (L35077), *Phytomonas* sp. Hart 1 (L35076); *Leishmania major* (AL354512), *Leishmania tarentolae* (AF016249), *Crithidia fasciculata* (J03470), *Phytomonas* sp. EM1 (AF243338), *Phytomonas* sp. Hart2 (AF243348), *H. pessoai* (X62331), *T. cruzi* (M59503), *T. avium* (AJ250736), and *Bodo saltans* (AF288757).

Nucleotide sequence accession number

The nucleotide sequence of the 5S rRNA gene of *H. ztiplika* n. sp. has been deposited at the GenBank and has the accession number AY308759.

RESULTS

Light microscopy observations

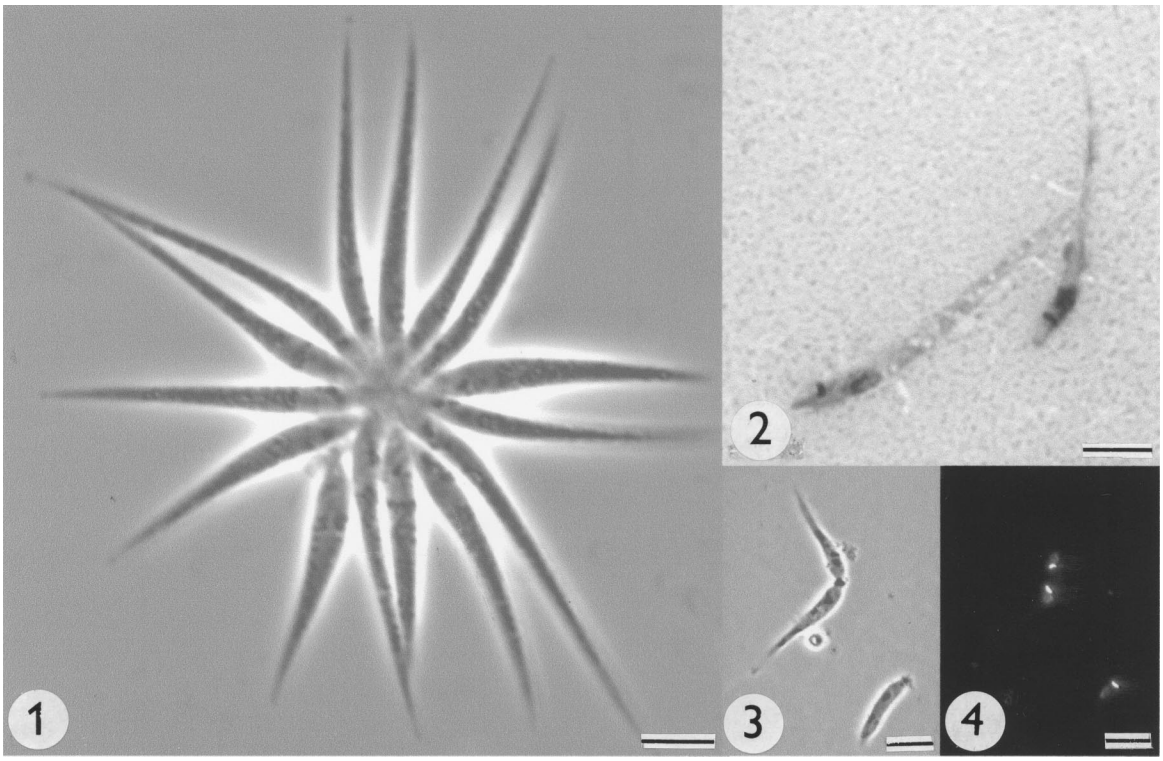
Dissection and light microscopic examination of 1,320 biting midges belonging to several species of *Culicoides* revealed kinetoplastid infections in about 1% of the specimens. In the hindgut and the Malpigian tubes, flagellates appeared to be free in the lumen or attached to the wall of the hindgut. Trypanosomatids isolated from a heavily infected female *C. kibunensis* (2% of the 280 examined females of this species harbored an infection) were inoculated into the SNB-9 culture medium and after several passages achieved density of 2×10^7 cells/ml within 6 days. This culture was identified as ICUL/CZ/1999/CER1 and studied in detail. Only cells characterized by an elongated body with an anterior prenuclear kinetoplast, considered to be promastigotes, were observed (Figs. 1–4). The flagellum originated near the kinetoplast and usually emerged from the side of the body, close to its anterior end. Giemsa-stained cells (Fig. 2) measured from 20.4 to 37.4 μm in length ($\bar{x} \pm$ SE: 28.1 ± 4.70 μm), whereas the breadth of the body at the level of the nucleus varied from 2.7 to 5.3 μm (3.6 ± 0.53 μm). The oval nucleus is situated in the anterior or central part of the cell. The distance between the anterior end of the body and the kinetoplast ranges from 2.0 to 3.7 μm (2.9 ± 0.50 μm), and the distance between the kinetoplast and the nucleus varies between 0.3 and 2.0 μm (1.1 ± 0.46 μm). The kinetoplast index (anterior end to kinetoplast/kinetoplast to nucleus) of 2.64 ± 0.55 μm indicates that the kinetoplast is closer to the nucleus than to the anterior end of the body. The posterior end is usually drawn out to a fine point. The cells appeared to be free of endosymbiotic bacteria.

The cells formed large and conspicuous rosettes in culture (Fig. 1), and agile promastigotes were apparently connected with each other in their anterior part. In an attempt to transform the promastigote stages into the opistomastigotes, according to the protocol described for *Herpetomonas* spp. (Roitman et al., 1976; Faria-e-Silva et al., 1996), the standard cultivation temperature of 23 C was increased to temperatures ranging between 30 and 33 C. The cells remained alive at 31 C, but at higher temperatures they ceased dividing, and the culture eventually died. No morphological change other than the rounding up of moribund cells has been observed under these conditions (not shown). Because DAPI staining allows relatively easy scoring of the mutual positions of kinetoplast and nucleus (Figs. 3, 4), it is unlikely that even at low prevalence, opistomastigotes have been overlooked.

Electron microscope observations

Cells are bound by a typical pellicular membrane, below which a series of subpellicular microtubules is located (Figs. 5–9). They measure 20 nm in outside diameter and are in regular rows about 19 nm apart (Fig. 7). The flagellum is contained in a deep flagellar pocket (Figs. 6, 9) and includes the familiar axoneme (9 + 2) complex. In sections of the flagellum after it has emerged from the flagellar pocket, the flagellar membrane enclosing the axoneme and paraxial rod becomes detached from these structures (Fig. 6). This morphologic transformation of the anterior flagellum results in the formation of numerous pseudopodium-like structures that contain finely fibrous dense material (Figs. 8, 9). These long, branched, fingerlike projections either become randomly intermingled or actively and extensively interdigitate, with projections of the flagella of other epimastigotes holding individual cells together in the form of rosettes (Figs. 9, 10).

The kinetoplast of *H. ztiplika* n. sp. is located between the flagellar pocket and the nucleus. It has a low-pitched and elongated shape, with DNA strands packed in parallel to the axis of the disk. The kinetoplast cylinder displays a single, centrally located electron-dense stripe, and the thickness of the kinetoplast is 0.16 ± 0.07 μm (Fig. 6).



FIGURES 1–4. Light microscopy of *Herpetomonas ztiplika* n. sp. **1.** Interference contrast of a large rosette. **2.** Giemsa-stained cells. **3, 4.** Same cells in phase contrast and DAPI stained. Bar = 5 μm (Figs. 1, 2) and 10 μm (Figs. 3, 4).

Phylogenetic analysis

An early phylogenetic analysis of the flagellate under study (designated as CER1) based on the 18S rRNA gene was performed to demonstrate that it was not a species of *Trypanosoma* (Votýpka et al., 2002). To analyze its phylogenetic position in more detail, we have extended the 18S rRNA alignment (1,892 bp long with 511 bp of ambiguously aligned nucleotides removed) by including 21 representatives of all available trypanosomatid genera and major clades identified thus far (Merzlyak et al., 2001). The studied trypanosomatid clusters within the endosymbiont-free *Herpetomonas* branch. All methods used strongly support its affiliation with this clade, with *H. pessoai* being its closest relative (Fig. 11). Major branches are well supported, and the branching order is not influenced by the use of different outgroups.

The 5S rRNA gene was aligned with 10 trypanosomatid species (only 4 insect trypanosomatid 5S rRNA genes are available), generating an alignment 146 bp long, which contained 40 parsimony-informative positions. Regardless of whether the Tv–Ts ratio was set to 1:2 to 1:5, the obtained tree revealed the monophyly of *H. ztiplika* n. sp. with *H. pessoai*, the only other available *Herpetomonas* species for the 5S rRNA data set, a relationship supported by 100% bootstrap by all methods used (Fig. 12). On the basis of the available sequence data, we conclude that this is a new species that belongs to *Herpetomonas*.

Taxonomic summary

Host: Culicoides kibunensis Tokunaga, 1937.

Type locality: Oak–hornbeam forest in the vicinity of Mikulov, Southern Moravia, Czech Republic.

Site of infection: Hindgut and Malpigian tubes.

Developmental stages: Promastigotes.

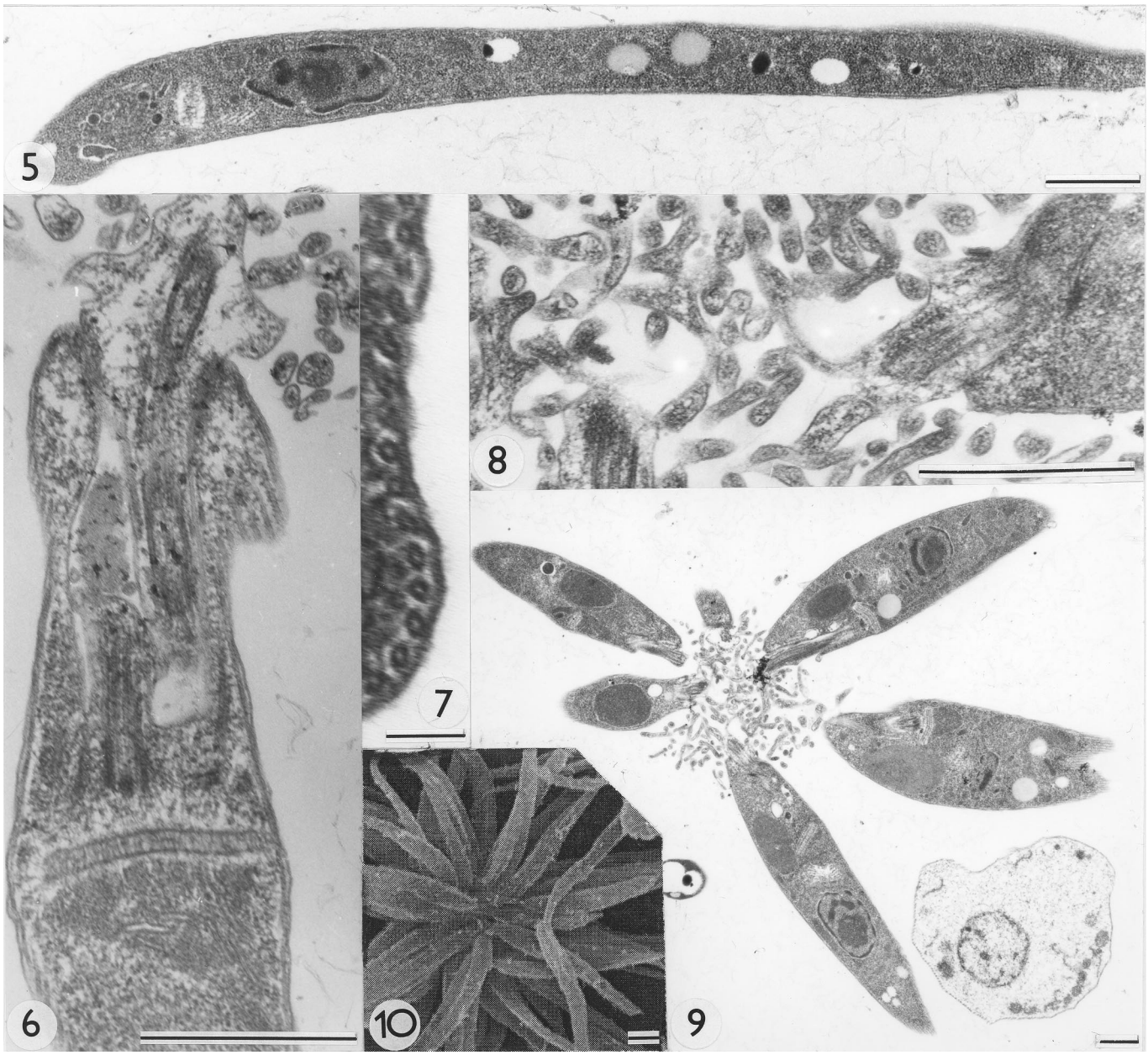
Type slides and stocks: Strain ICUL/CZ/1999/CER1; deposited in the type collection of the Faculty of Sciences, Charles University, Prague, Czech Republic.

Etymology: The species name is derived from the Czech name of the host insect, meaning biting midge. It is used as a noun in apposition.

DISCUSSION

Protozoan parasites of the ceratopogonid flies have not been subject to a thorough study. To our knowledge, the new kinetoplastid species described herein represents the first case of trypanosomatid infection in these insects, the abundance of which in the vast regions of the Northern Hemisphere is notorious.

Initial morphological study of the protozoan was inconclusive as to its assignment to a given trypanosomatid genus because of the lack of any characteristic morphological features. A feature worth mentioning is the abundant presence of rosettes, which is a typical trypanosomatid feature, with cells attached to each other as previously described for *Leptomonas peterhoffi* (Frolov and Skarlato, 1990). Therefore, we resorted to a phylogenetic analysis of 2 ribosomal RNA genes amplified from the culture stages; 18S rRNA was the first gene of choice because it is already available from dozens of trypanosomatids, including representatives of all known genera. Inclusion of the obtained sequence in the 18S rRNA alignment revealed the affiliation of the species under study with the insect trypanosomatids and the endosymbiont-free *Herpetomonas* species in



FIGURES 5–10. Transmission and scanning electron microscopy of *Herpetomonas ztiplika* n. sp. **5.** Typical elongated promastigote. **6.** Anterior part of the cell with a thin kinetoplast disk and deep flagellar pocket. **7.** Cross-sectioned corset of subpellicular microtubules. **8.** Numerous pseudopodium-like structures of the anterior flagellum. **9.** Section through a rosette revealing tiny projections of the flagella that hold them together. **10.** Large rosette containing dozens of promastigotes. Bar = 1 μm (Figs. 5, 6, 8, 9), 100 nm (Fig. 7), and 5 μm (Fig. 10).

particular. Further analyses of the 18S rRNA data set by various methods showed that this relationship was strongly supported.

The 5S rRNA gene is considered to be particularly useful to distinguish between closely related kinetoplastids (Fernandes et al., 1997; Dollet et al., 2000). To complement the 18S rRNA–based phylogeny, we sequenced the 5S rRNA gene repeat and aligned it with the only four 5S rRNA sequences from insect trypanosomatids deposited in the GenBank to date. Still, a monophyly of *H. ztiplika* n. sp. with *H. pessoai*, the only other *Herpetomonas* species available, was supported by 100% bootstrap. Generally, the 18S and 5S rRNA–based trees were in good correlation and pointed to the appurtenance of the kinetoplastid studied to the endosymbiont-free herpetomonads.

Furthermore, we isolated kDNA according to the protocol described elsewhere (Votýpka et al., 2002). Restriction analysis of the kDNA network revealed that the minicircles of *H. ztiplika* n. sp. are of uniform size, 1.3 kb (data not shown), which is in good correlation with the kinetoplast thickness, measured by electron microscopy (Lukeš and Votýpka, 2000). Minicircles of a similar small size have been described in other species of *Herpetomonas* that do not contain symbiotic bacteria (Hollar et al., 1998). It should be noted, however, that small minicircles

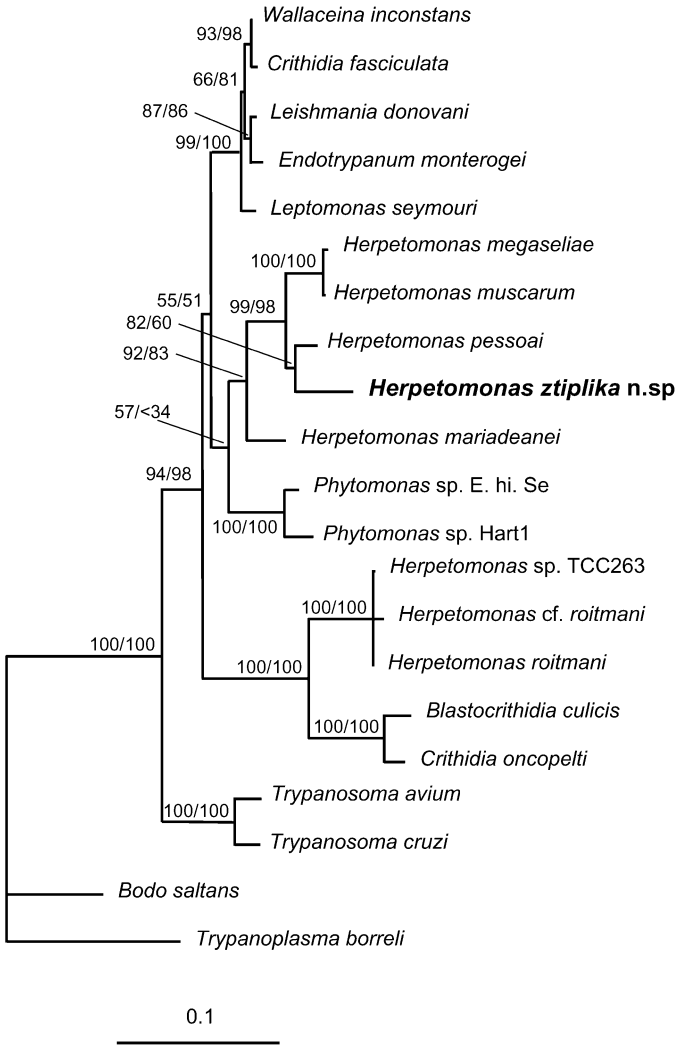


FIGURE 11. ML tree of the 18S rRNA gene sequences of trypanosomatids rooted at *Bodo saltans* and *Trypanoplasma borreli* (−ln = 7963.8377, α shape parameter = 0.69071). Bootstrap values (ML; MP, Tv–Ts = 1:2) are indicated. The distance scale is given under the tree.

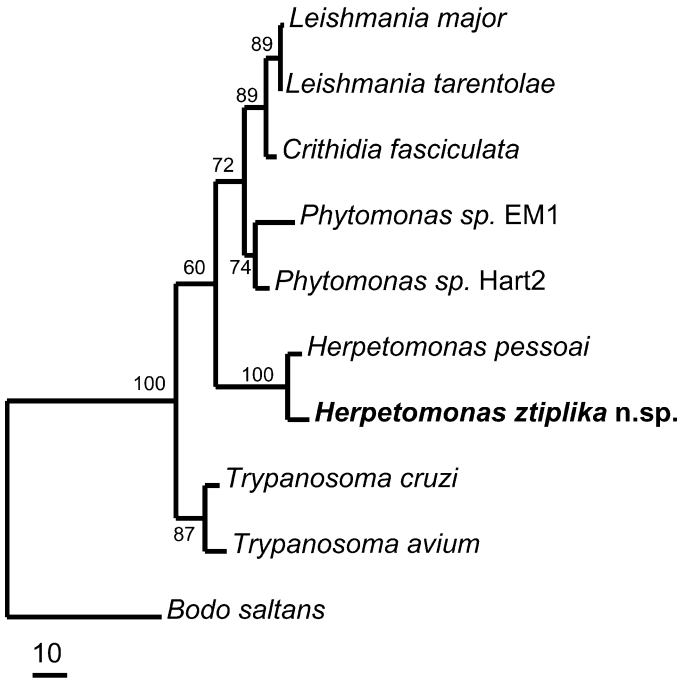


FIGURE 12. MP tree of the 5S rRNA gene sequences of trypanosomatids rooted at *Bodo saltans* (Tv–Ts = 1:2, 189 steps). Bootstrap values are indicated. The distance scale is given under the tree.

have also been found in some *Phytomonas* and *Leptomonas* species (Kolesnikov et al., 1990; Ahomadegbe et al., 1992). Two other features, the even spacing of subpellicular microtubules and the absence of peripheral mitochondrial branches, are shared between symbiont-free trypanosomatids and the flagellate under study (Freymuller and Camargo, 1981; De Souza and Motta, 1999).

To confirm this relationship on a different level, we have carefully evaluated the morphology of the promastigotes. This was the only stage observed in culture. It has the posterior end drawn out to a fine point, a feature specific to herpetomonads according to Wallace (1966). For the majority of *Herpetomonas* species described so far, just a handful of morphological characters (body length, body width, and length of the flagellum) are available and can be used for differential diagnosis. When these are considered, *H. ztiplika* n. sp. belongs to a large herpetomonad group, with only 2 species (*H. siphunculinae* and *H. ludwigi*) attaining a similar size (Patton, 1921; Kramář, 1950).

Because all morphological features mentioned above have also been observed in other trypanosomatids, we searched for the main character of *Herpetomonas*, the opistomastigote stage (Wallace, 1966; Vickerman, 2000). Enhancement of the cultivation temperature, which resulted in the transformation of promastigotes into opistomastigotes in some *Herpetomonas* species (Faria-e-Silva et al., 1996), did not have a similar effect in the culture of *H. ztiplika* n. sp. Therefore, in our opinion, the presence of an opistomastigote stage can no longer be considered as a prerequisite for placing a new flagellate species in the genus *Herpetomonas*.

The sequence and morphological data generated are consistent with the protozoan studied being a novel endosymbiont-free trypanosomatid, which we have assigned to *Herpetomonas*. The description of *H. ztiplika* n. sp. was based primarily on the 18S and 5S rRNA sequence data because the morphological features available are insufficient for a clear-cut generic diagnosis. Our results indicate that part of the midge population harbors flagellates that are likely to be specific for these economically important insects. We were able to establish a culture of these parasites, making them amenable for future studies.

ACKNOWLEDGMENTS

We thank Ivan Fiala (Institute of Parasitology) for help with molecular phylogeny. This work was supported by the Ministry of Education, grant MSM123100003 (to J.L.); the Grant Agency of Charles University, grant 147/2002/B-BIO (to J.V. and M.S.); and the Centre de Coopération Internationale en Recherche Agronomique pour le Développement (CIRAD), grant ATP 2002/30 (to S.P.).

LITERATURE CITED

AHOMADEGBE, J.-C., M. DOLLET, AND G. RIOU. 1992. Kinetoplast DNA from plant trypanosomatids responsible for the Hartrot disease in coconut trees. Biology of the Cell **74:** 273–279.

CERISOLA, J. A., C. F. DEL PRADO, R. ROHWEDDER, AND J. P. BOZZINI. 1971. *Blastocrithidia triatoma* n. sp. found in *Triatoma infestans* from Argentina. Journal of Protozoology **18:** 503–506.

DEDET, J.-P., AND F. PRATLONG. 2000. Leishmania, Trypanosoma and monoxenout trypanosomatids as emerging opportunistic agents. Journal of Eukaryotic Microbiology **47:** 37–39.

DE SOUZA, W., AND M. C. M. MOTTA. 1999. Endosymbiosis in protozoa of the Trypanosomatidae family. FEMS Microbiology Letters **173:** 1–8.

DOLLET, M., N. R. STURM, M. SANCHEZ-MORENO, AND D. A. CAMPBELL. 2000. 5S ribosomal RNA gene repeat sequences define at least eight groups of plant trypanosomatids (*Phytomonas* spp.): Phloem restricted pathogens form a distinct section. Journal of Eukaryotic Microbiology **47:** 569–574.

FARIA-E-SILVA, P. M., M. J. SOARES, AND W. DE SOUZA. 1996. Proliferative opistomastigotes forms in *Herpetomonas roitmani* (Kinetoplastida: Trypanosomatidae). Parasitology Research **82:** 125–129.

FERNANDES, O., M. M. G. TEIXEIRA, N. R. STURM, M. A. SOUSA, E. P. CAMARGO, W. M. DEGRAVE, AND D. A. CAMPBELL. 1997. Mini-exon gene sequences define six groups within the genus *Crithidia*. Journal of Eukaryotic Microbiology **44:** 535–539.

FREYMULLER, E., AND E. P. CAMARGO. 1981. Ultrastructural differences between species of trypanosomatids with and without endosymbionts. Journal of Protozoology **28:** 175–182.

FROLOV, A. O., AND S. O. SKARLATO. The structure of rosette-like cell associations in the lower trypanosomatids. Parasitoilogija (Leningrad). **32:** 455–461. [In Russian with English summary.]

HOLLAR, L., J. LUKEŠ, AND D. A. MASLOV. 1998. Monophyly of endosymbiont containing trypanosomatids: Phylogeny versus taxonomy. Journal of Eukaryotic Microbiology **45:** 293–297.

KOLESNIKOV, A. A., D. A. MASLOV, AND S. A. PODLIPAEV. 1990. Comparative restriction enzyme cleavage analysis of kinetoplast DNA from the lower trypanosomatids isolated in the North-West region of the USSR. Archiv fur Protistenkunde **138:** 239–250.

KRAMÁŘ, J. 1950. Parasites of the larva of *Tipula maxima* Poda. Bulletin of the Czechoslovak Zoological Society **14:** 55–76. [In Czech with English summary.]

LUKEŠ, J., AND J. VOTÝPKA. 2000. *Trypanosoma avium*: Novel features of the kinetoplast structure. Experimental Parasitology **96:** 178–181.

MERZLYAK, E., V. YURCHENKO, A. KOLESNIKOV, K. ALEXANDROV, S. PODLIPAEV, AND D. MASLOV. 2001. Diversity and phylogeny of insect trypanosomatids based on small subunit rRNA genes: Polyphyly of *Leptomonas* and *Blastocrithidia*. Journal of Eukaryotic Microbiology **48:** 163–171.

MILTGEN, F., AND I. LANDAU. 1982. *Culicoides nubeculosus*, an experimental vector of a new trypanosome from Psittaciform: *Trypanosoma bakeri* n. sp. Annales de Parasitologie Humaine et Comparée **57:** 423–428.

NOYES, H., F. PRATLONG, M. CHANCE, J. ELLIS, G. LANOTTE, AND J. P. DEDET. 2002. A previously unclassified trypanosomatid responsible for human cutaneous lesions in Martinique (French West Indies) is the most divergent member of the genus *Leishmania ss*. Parasitology **124:** 17–24.

NUNES, L. R., M. M. G. TEIXEIRA, E. P. CAMARGO, AND H. T. AFFONSO. 1994. kDNA and rDNA sequences reveal a phylogenetic cluster of species originally placed in different genera of trypanosomatids. Journal of Eukaryotic Microbiology **41:** 496–500.

PATTON, W. S. 1921. Studies on the flagellates of the genera *Herpetomonas*, *Crithidia* and *Rhynochoidomonas*. The morphology and life history of *Herpetomonas siphunculinae* sp. nov., parasitic in the alimentary tract of *Siphunculina funiculai*. Indian Journal of Medical Research **8:** 613–620.

PODLIPAEV, S. A. 1990. Catalogue of world fauna of Trypanosomatidae (Protozoa). Proceedings of the Zoological Institute, Leningrad **144:** 1–178.

———. 2000. Insect trypanosomatids: The need to know more. Memorias Do Instituto Oswaldo Cruz **95:** 517–522.

POSADA, D., AND K. A. CRANDALL. 1998. Modeltest: Testing the model of DNA substitution. Bioinformatics **14:** 817–818.

ROITMAN, I., Z. BRENER, V. C. ROITMAN, AND E. W. KITAJIMA. 1976. Demonstration that *Leptomonas pessoai* Calvao, Oliveira, Carvalho & Veiga, 1970, is a *Herpetomonas*. Journal of Protozoology **23:** 291–293.

SWOFFORD, D. L. 2001. PAUP*: Phylogenetic analysis using parsimony, version 4.0b10. Sinauer Associates, Sunderland, Massachusetts.

VICKERMAN, K. 2000. Order Kinetoplastida Honigberg, 1963. *In* The illustrated guide to the Protozoa, J. J. Lee, G. F. Leedale, and P. Bradbury (eds.). Society of Protozoologists, Lawrence, Kansas, p. 1159–1184.

VOTÝPKA, J., M. OBORNÍK, P. VOLF, M. SVOBODOVÁ, AND J. LUKEŠ. 2002. *Trypanosoma avium* of raptors (Falconiformes): Phylogeny and identification of vectors. Parasitology **125:** 253–263.

WALLACE, F. G. 1966. The trypanosomatid parasites of insects and arachnids. Experimental Parasitology **18:** 124–193.