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Life cycle of *Blastocrithidia papi* sp. n. (Kinetoplastea, Trypanosomatidae) in *Pyrrhocoris apterus* (Hemiptera, Pyrrhocoridae)

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Abstract

Blastocrithidia papi sp. n. is a cyst-forming trypanosomatid parasitizing firebugs (*Pyrrhocoris apterus*). It is a member of the *Blastocrithidia* clade and a very close relative of *B. largi*, to which it is almost identical through its SSU rRNA gene sequence. However, considering the SL RNA gene these two species represent quite distinct, not even related typing units. Morphological analysis of the new species revealed peculiar or even unique features, which may be useful for future taxonomic revision of the genus *Blastocrithidia.* These include a breach in the microtubular corset of rostrum at the site of contact with the flagellum, absence of desmosomes between flagellum and rostrum, large transparent vacuole near the flagellar pocket, and multiple vacuoles with fibrous content in the posterior portion of the cell. The study of the flagellates' behavior in the host intestine revealed that they may attach both to microvilli of enterocytes using swollen flagellar tip and to extracellular membranes layers using hemidesmosomes of flagellum. Laboratory experiments on *B. papi* transmission in *P. apterus* demonstrated that the parasite may be transmitted vertically (via contaminated surface of eggs) and horizontally (via contaminated substrate and/or necrophagy). We argue that the parasite exploits transmission mechanisms intended for obligate bacterial symbionts of *P. apterus.*

Keywords: Cyst-forming trypanosomatids; Firebugs; Parasites; Transmission; Ultrastructure

Introduction

Family Trypanosomatidae (Doflein, 1901) Grobben 1905 is one of the most intensively studied groups of protists. The research attention is mostly attracted by dixenous (two hosts) *Trypanosoma* spp. and *Leishmania* spp. which are economically and/or medically important since they parasitize humans and livestock. However, the vast majority of trypanosomatids are monoxenous (one host) parasites of insects and have been generally neglected for a long time (Maslov et al. 2013). In the last years, monoxenous trypanosomatids have more and more frequently become objects of study. The increased interest in this group is justified by its importance for understanding the origin and evolution of dixeny and par-

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asitism per se (Flegontov et al. 2016; Kraeva et al. 2015, 2016; Lukeš et al. 2014). Some of these parasites bear intracytoplasmic bacteria, which were acquired at different times and from different sources and therefore present a good model to study the evolution of endosymbiosis (Kostygov et al. 2016; Teixeira et al. 2011; Votýpka et al. 2014). Recently one group of monoxenous trypanosomatids was demonstrated to evolve a non-canonical genetic code with all three termination codons reassigned as sense ones (Zahonova et al. 2016). Monoxenous trypanosomatids also proved to have impact on population dynamics and fitness of their hosts (Hamilton et al. 2015; Kozminsky et al. 2015; Votýpka et al. 2012b).

Trypanosomatid morphology is conservative even under the electron microscope (Frolov 2000; Frolov and Karpov 1995; Vickerman 1976) and it is difficult or impossible to find unique ultrastructural features suitable for taxonomical purposes. As a result, morphology is usually considered unsuitable for trypanosomatid systematics, and morphological analysis in taxonomical descriptions is often quite superficial. This complicates comparative analysis of new species and decreases the chances to discover synapomorphies for the taxa of various ranks, the number of which is constantly growing (Maslov et al. 2013).

For the moment, the family Trypanosomatidae includes 4 formally described subfamilies: Leishmaniinae (*Leishmania*, *Leptomonas*, *Crithidia*, *Lotmaria*, and *Novymonas*), Phytomonadinae (*Phytomonas*, *Herpetomonas*, and *Lafontella*), Blechomonadinae (*Blechomonas*), and Strigomonadinae (*Strigomonas*, *Angomonas*, and *Kentomonas*) (Jirků et al. 2012; Votýpka et al. 2013, 2014; Yurchenko et al. 2016). In addition, there are several genus-level lineages (including uncharacterized ones) yet unassigned to subfamilies (Votýpka et al. 2015).

An example of such a clade is a group historically named "cyst-forming trypanosomatids" (Kostygov and Frolov 2007; Podlipaev and Frolov 2000). The apomorphic trait for these species is a presence of specialized aflagellate cells ("cysts"), which are used to facilitate the transmission and long-term survival in unfavorable conditions (Dias Fde et al. 2014; Gibbs 1950; McGhee and Cosgrove 1980). The group unites representatives of the genus *Blastocrithidia* and a sister clade composed of species related to *Leptomonas jaculum* (Kostygov and Frolov 2007; Votýpka et al. 2012a). Cyst-forming trypanosomatids are known to be difficult to cultivate (Maslov et al. 2010; Peng and Wallace 1981; Reduth et al. 1989), and some GenBank entries initially attributed to these species were later found to belong to other trypanosomatids from original mixed infections of the hosts (Kostygov et al. 2014; Yurchenko et al. 2009).

Firebugs *Pyrrhocoris apterus* are widely distributed and easily cultivated colonial insects being used as a model object in various biological studies (Socha 1993). High density of a local population and longstanding exploitation of one biotope predetermine stable parasitic infections in the colonies of these bugs. Recent broad-scale survey of trypanosomatid fauna in Pyrrhocoridae demonstrated *Leptomonas pyrrhocoris* Zotta, 1912 (subfam. Leishmaniinae) to be the predominant species in all tested European populations. Besides, there were four other "molecular species" of trypanosomatids detected: TU59, TU71 (described as *L. scantii*, Leishmaniinae), TU73, and TU74. TU73 remained uncharacterized, whereas TU59 (=Ch2) and TU74 proved to belong to the *Blastocrithidia* clade (Votýpka et al. 2012b).

Earlier we discovered an invasive colony of firebugs north of the Pskov region in Russia (Frolov et al. 2014). The insects in this colony were infected with two species of trypanosomatids, one of which was identified as *L. pyrrhocoris*, whereas the second was a novel cyst-forming species. Here we performed a morphological and molecular characterization of this new species, described details of its life cycle, and disclosed mechanisms of its transmission in the host.

Material and Methods

Insect hosts and field work

Firebugs *Pyrrhocoris apterus* were collected from a naturally-infected 3 y.o. colony north of the Pskov region, Russia (58°35′N; 28°55′E) (Frolov et al. 2014). The core of this colony was found on 2 linden (*Tilia cordata*) trees (~65 y.o.) while individual bugs were encountered on the surrounding meadow (~900 m^2).

The annual developmental cycle in this colony consisted of the 3 stages characterized earlier (Koštál and Šimek 2000): i) long-term diapause (D) lasting up to 7 months (from mid-October till May); ii) generation P1 from the eggs of overwintered females D, laid in the first half of May; and iii) generation P2 from the eggs of P1 females, laid between the end of July and the beginning of August. The imagines of P2 and, partially, P1 go into the diapause. Trypanosomatids were investigated in each of the three developmental stages – D, P1, and P2. Insects were collected by hand and either analyzed immediately or cultured as described previously (Kaltenpoth et al. 2009).

Dissection of bugs

The insects were euthanized by chloroform, and a drop of haemolymph from a leg was investigated by dark-field light microcopy using Leica DM 2500 (Leica Microsystems, Wetzlar, Germany) as described before (Frolov et al. 2016b). The dissection of the gut and the salivary glands was done in normal saline solution in accordance with conventional practice and classification (Haas and Konig 1987). Gut's fragments with identified flagellates were smeared for microscopy, and used for establishing cultures and DNA isolation.

Cultivation of trypanosomatids

Xenic culture of the isolate Bl_Pa3 was obtained on the biphasic blood agar medium containing 3 ml of Brain Heart

Infusion, BHI (BD, Franklin Lakes, USA) with 2% agar (Difco Laboratories, Detroit, USA) and 10% inactivated human blood overlaid with 2 ml of the liquid media – BHI and 10% of the fetal bovine serum (BioloT, St. Petersburg, Russia). Liquid phase was supplemented with 500 μg/ml of streptomycin and 500 Units/ml of penicillin. Cultures were maintained at 20 °C and passaged monthly. All attempts to establish an axenic culture of the isolate Bl_Pa3 using the previously described device (Podlipaev and Frolov 1987) were unsuccessful. Flagellates isolated from the "clean" part of the apparatus and seeded to blood agar medium stopped dividing and ultimately died, whereas the original culture containing mycelial fungi effectively propagated.

Light microscopy

Smears were air-dried on slides and fixed with ethanol for 30 min. Giemsa staining was performed according to the previously described protocols (Jirků et al. 2012). Digital images of cells were obtained using Leica DM 2500 light microscope equipped with UCMOS14000KPA 14-Mpx camera. Cell measurements (n = 25) and statistical analysis were carried out by UTHSCSA Image Tool for Windows v. 3.0 software (http://compdent.uthscsa.edu/dig/itdesc.html).

Transmission electron microscopy

Bugs' intestinal fragments and flagellates collected by centrifugation from the culture were prepared for transmission electron microscopy (TEM) and observed as described earlier (Frolov et al. 2016b).

DNA isolation, amplification, cloning and sequencing

Total genomic DNA was isolated from the field samples using either salt extraction protocol (Aljanabi and Martinez 1997) or with the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. SSU rRNA gene was amplified using either S762 and S763 (Maslov et al. 1996), or 1127F and 1958R (Kostygov and Frolov 2007) primer pairs, following the previously described protocol (Losev et al. 2015). These PCR fragments were sequenced directly as reported elsewhere (Kostygov et al. 2011). 5S/SL RNA repeat region was amplified with the primers M167 and M168 (Westenberger et al. 2004), and then cloned using InsTA PCR Cloning Kit (Thermo Fisher Scientific, Waltham, USA). 4 clones were sequenced for each of 8 samples. The GenBank accession numbers for the new sequences determined in this work are KX641338–KX641340 (SSU rRNA gene) and KX641341–KX641373 (5S/SL RNA repeat region).

Phylogenetic analyses

The alignment of 53 SSU rRNA gene sequences of trypanosomatids, representing known phylogroups of these flagellates was prepared as described before (Gerasimov et al. 2012). Maximum likelihood analysis was performed in IQTREE v. 1.4.1 (Nguyen et al. 2015) under TIM3e + I + G4 model (as selected by the program) using "standard" bootstrap test with 1000 replicates. Bayesian inference was completed in MrBayes 3.2.6 (Ronquist et al. 2012) under GTR + I + G model (4 gamma categories) with analysis run for 5 million generations and sampling every 1000th of them. Other parameters were set as default.

5S/SL RNA gene sequences were aligned with the use of MAFFT v. 7 (Katoh and Standley 2013). The resulting alignment was manually refined in Bioedit (Hall 1999), then non-conserved and ambiguously aligned positions were removed using Gblocks (Castresana 2000) as described elsewhere (Chistyakova et al. 2014). The sequences were clustered in MEGA7 software using neighbor-joining method, K2P model, pairwise gap deletion and 1000 bootstrap replicates.

Determination of modes of transmission

Vertical transmission

One hundred fifty eggs from seven clutches were collected in May 2014 on the territory of the natural colony of *P. apterus*. The clutches were located at a depth of 1.5–3 cm in sand molehills. The eggs were rolled several times on a filter paper to clean them from extraneous particles and divided into 2 groups. The eggs of group A were distributed among three sterile cages, equipped with drinking bowls and grinded linden seeds. Analogous cages were used for group B, but in this case the eggs' surface was sterilized by intensive washing of bunches of 20–30 eggs in net boxes according to the following scheme: dH_20 – 5 min, 95% ethanol – 5 min, dH_20 – 5 min, drying on filter paper. The bugs of both groups were dissected on days 4, 12 and 20 after hatching, i.e. on nymphal stages 1, 3 and 4–5. Every time, 10 individuals randomly taken from the respective cages were used. The experiment was repeated with the progeny of the remaining bugs of the group A following the same protocol. The eggs laid by these bugs were also divided into two groups: (A2 and B2). Analogously, the surface of B2 eggs was sterilized.

Horizontal transmission via contaminated substrate

In this experiment imagines of *P. apterus* of the group A (infected with *B. papi*) and nymphs of stages 3–4 of the group B2 (parasite-free) were used. Nymphs of B2 were further divided into the groups C1 and C2, each containing 20 individuals. C1 was incubated for one week together with 10 imagines of group A, then the former were placed into a sterile cage. Group C2 served as a control and was kept in a sterile cage. Upon separation of C1 from A, 10 randomly

selected bugs from the groups C1 and C2 were dissected. The remaining individuals were dissected 7 days later. The experiment was carried out twice.

Horizontal transmission via necrophagy

This experiment was carried out in the same way as the previous one, except that imagines of group A were euthanized with chlorophorm one day earlier. Moreover, to promote necrophagy the nymphs had no other food during the experiment and had starved for 5 days before it. The two groups of nymphs were analogously designated as N1 (experimental) and N2 (control).

Results

Phylogenetic analyses

SSU rRNA sequences of the isolates from Pskov region (Pa1 and Pa3) were identical to each other and to that of the isolate 531Py obtained in 2009 from *P. apterus* in the Orenburg region. The comparison with the sequences from Genbank revealed the new species to be different from *Blastocrithidia largi* (first BLAST hit) only by two substitutions in the V7 segment of the gene.

Maximum likelihood and Bayesian trees based on this marker were generally congruent except for a few branches with low support (Fig. 1). The new species nested within the *Blastocrithidia* clade, specifically in the main subclade composed of *B. largi* (the closest relative), *B. triatomae and B. cyrtomeni*. The statistical supports for the relationships within this subclade were rather low apparently due to small differences in the sequences as demonstrated by short branches (Fig. 1).

Therefore, we performed analysis with a less conservative marker – SL RNA gene repeats, which, in the case of *Blastocrithidia* spp., also contain 5S rRNA gene. SL RNA gene with its intergenic spacer is commonly used for trypanosomatid species delineation following the concept of typing units (TU) defined by an arbitrary threshold of 90% identity (Maslov et al. 2007; Westenberger et al. 2004). Direct comparison and cluster analysis of 5S/SL RNA repeats sequences (Fig. 2) demonstrated that 8 isolates from *P. apterus* collected in the Pskov region were indistinguishable from each other as well as from that previously collected in Moravia and attributed to TU74 (Votýpka et al. 2012b). The differences between molecular clones of any isolate were on the same scale as those between isolates. None of them exceeded 9.6%. These differences consisted mainly in the number of short tandem repeats, whereas single-nucleotide indels and substitutions composed less than 1.5% of them. *B. largi*, which was hardly distinguishable by SSU rRNA gene, proved to be identical to the trypanosomatid under study by only ~80% through the 5S/SL RNA repeat sequence. Moreover, it was not even the closest relative on the dendrogram built with the use of this marker (Fig. 2). The sister group for the new species' clade was TU59 (=Ch2) comprising one isolate from *P. apterus* (Slovakia) and two from predatory bugs (China). The differences between the members of the two clades ranged from 12 to 19%.

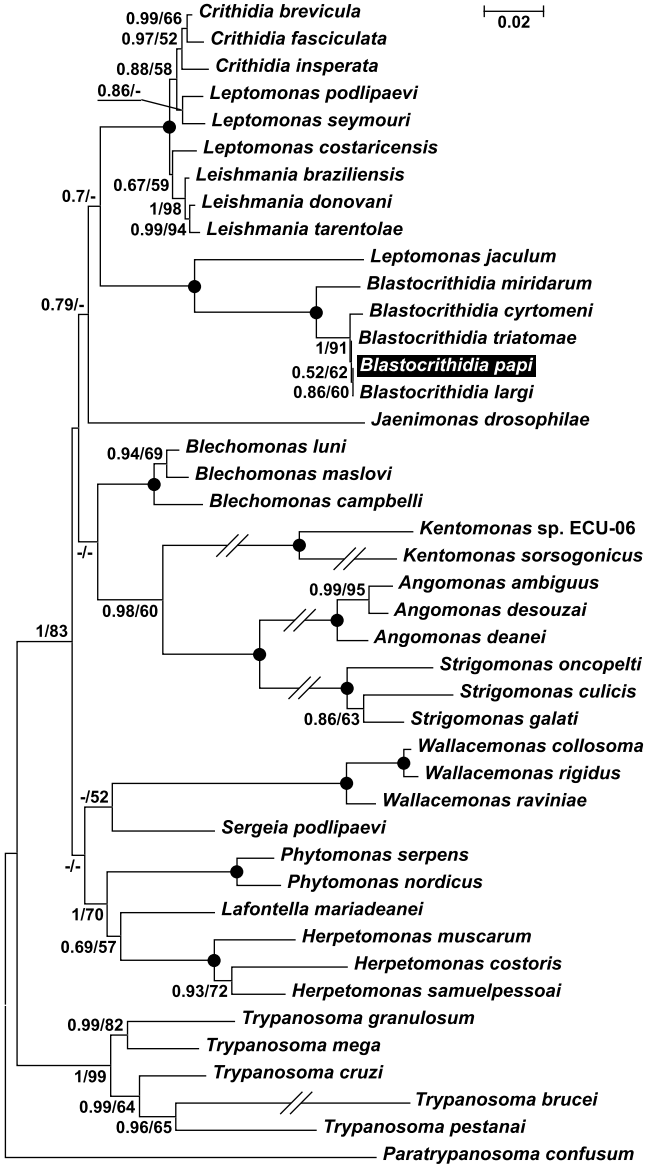

Fig. 1. Maximum likelihood phylogenetic tree reconstructed using 18S ribosomal RNA gene sequences. Numbers at nodes indicate posterior probability and bootstrap percentage, respectively. Values less than 0.5 and 50% are replaced with dashes. Nodes having 1.0 posterior probability, 100% bootstrap support are marked with black circles. Double-crossed branches are at 50% of their original lengths. The tree is rooted with the sequence of *Paratrypanosoma confusum*. The scale bar denotes the number of substitutions per site. The species under study (*Blastocrithidia papi*) is highlighted. Accession numbers of the sequences retrieved from GenBank are available upon request.

In sum, the sequence analysis demonstrated that the trypanosomatid under study should represent a separate species

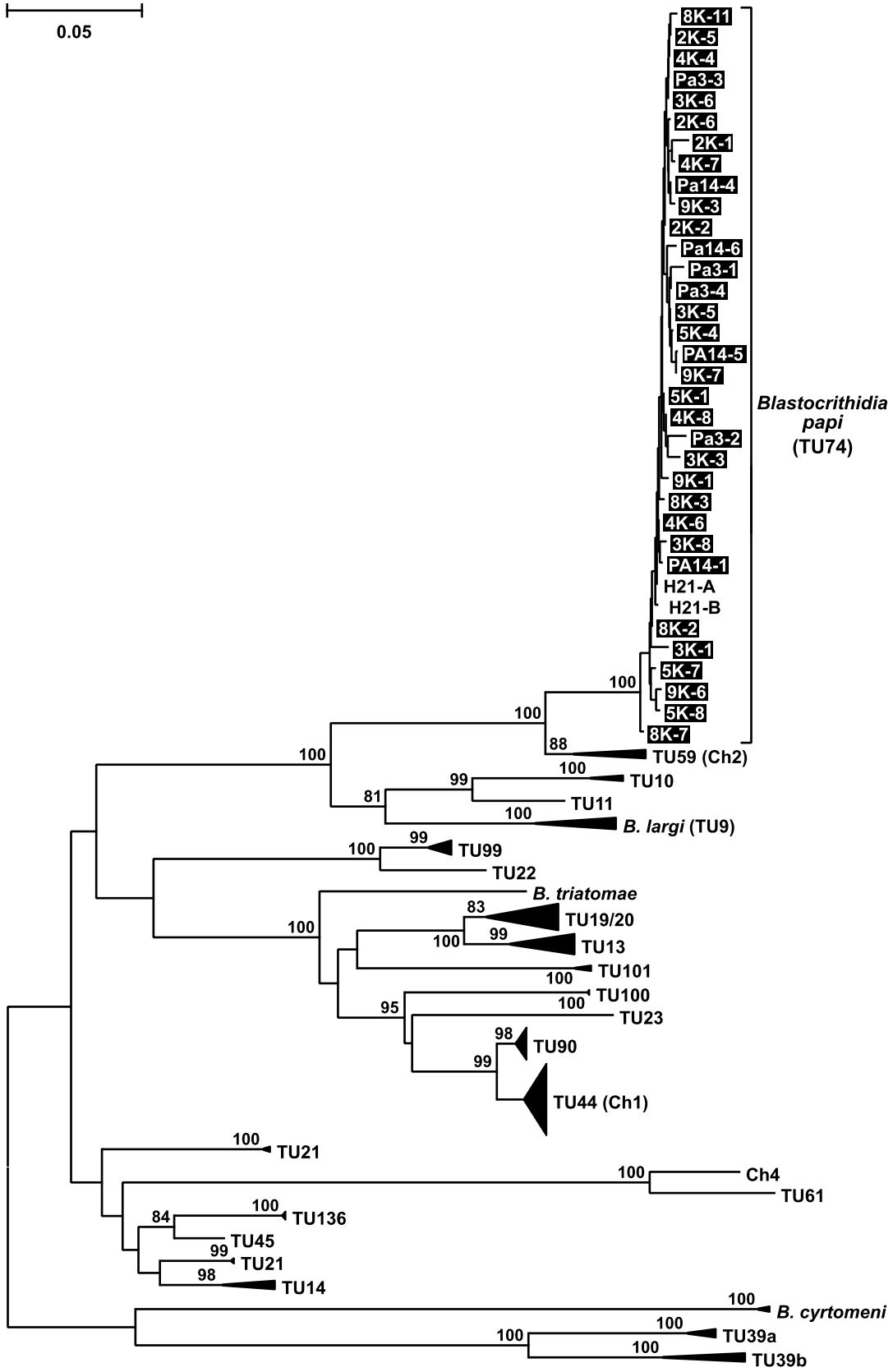

Fig. 2. Midpoint-rooted neighbor-joining dendrogram of SL RNA/5S rRNA gene sequences of *Blastocrithidia triatomae* subclade (see Fig. 1). All clades corresponding to typing units, except that of *B. papi* are collapsed Numbers at nodes indicate bootstrap percentage, values less than 70% are omitted. The isolates obtained in this work are highlighted. The bar represents the number of substitutions per site. Accession numbers of the sequences retrieved from GenBank are available upon request.

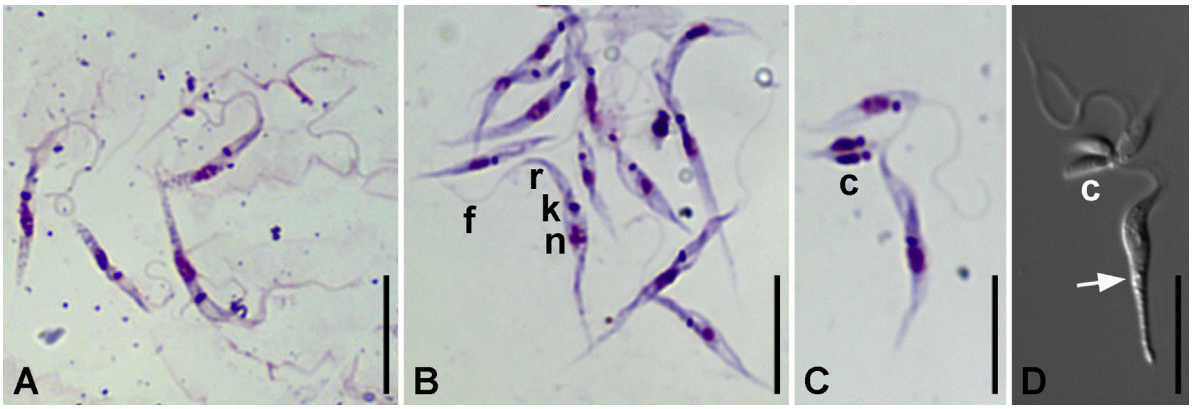

Fig. 3. Light microscopy of *Blastocrithidia papi* in the host intestine and in the culture. **A–C**, Giemsa-stained smears, bright field; **D**, Differential interference contrast. **A**, flagellates in M3 region of midgut of *P. apterus*; **B**, epimastigotes in culture; **C**, **D**, formation of cysts in Malpighian tubules. Arrow points to light-refracting granules in the posterior part of epimastigote. **f** – flagellum; **k** – kinetoplast; **n** – nucleus; **r** – rostrum; **c** – "cysts". Scale bars are 15 μm.

of *Blastocrithidia*. Hereafter it is referred to as *Blastocrithidia papi* sp. n.

Morphological characterization of *Blastocrithidia papi*

Light microscopy

Flagellates in the intestine and those in the culture of *B. papi* were morphologically indistinguishable (Fig. 3A, B). Epimastigotes of this species were fusiform with both anterior and posterior ends tapered and significantly elongated. The nucleus was located approximately in the center of the cell. Behind it, there were multiple vacuoles of various sizes. On Giemsa-stained smears, these structures appeared homogeneous, while in living cells they formed a chain of light-refracting granules (Fig. 3D). The anterior end of the cell had a "rostrum" measuring circa 1/3–1/2 of the total cell body length. The flagellum was attached to the rostrum and was approximately twice longer.

One of the characteristic traits of *Blastocrithidia papi* is the ability to form "cysts". This process went similarly in the host and in the xenic culture (Fig. 3C, D). Pre-cystic stages were attached to flagella of mother epimastigotes, whereas "cysts" maturated separately. In 60% of cyst-forming epimastigotes, there were three flagellum-associated daughter cells. One large cell was situated distally and two smaller ones were localized in the proximal part of the flagellum, close to the rostrum (Fig. 3C, D). Only one large or only two small daughter cells were present in 20% and 15% of epimastigotes, respectively. Less than 5% of epimastigotes bore on their flagella four small cells arranged in pairs. All measurements of daughter cells are summarized in Table 1. Formation of "cysts" was detected in the M3 section of the midgut (see below), and in the lumen of the Malpighian tubules of *P. apterus*.

Ultrastructure

The fine structure of *B. papi* cells was generally similar to that of other trypanosomatids. Most organelles were located in the anterior half of the cell (Fig. 4A). The laterally opened shallow flagellar pocket was filled with electron-dense granular content (Fig. 4A, B). Two basal bodies were situated between kinetoplast and flagellar pocket bottom (Fig. 4A). By the side of the flagellar pocket, there was a vacuole of irregular shape with transparent homogenous content (Fig. 4B). The external part of the flagellum was attached to the rostrum along the whole length of the latter, but no desmosomes were observed between them. The microtubular corset of the rostrum was breached at the site of contact with the flagellum (Fig. 4C). The kinetoplast (650 nm in diameter and 117 nm thick) was oriented transversally in relation to the longitudinal axis of the cell (Fig. 4A). Dictyosomes of the Golgi apparatus were present between nucleus and kinetoplast, (Fig. 4A). Sometimes there were also 1–2 large vacuoles bearing medium electron density content (Fig. 4A) with numerous fibrils 7–9 nm in diameter (not shown). Such vacuoles were abundant in the postnuclear zone (Fig. 4D) and, apparently, corresponded to the vacuoles/granules observed in light microscope (see above). Numerous glycosomes and branches of mitochondrion were spread throughout the cytoplasm (Fig. 4A, D).

The "cysts" of *B. papi* displayed typical features of such cells: electron-dense cytoplasm filled with ribosomes; DNA-containing organelles hardly discernible due to intense condensation of all structures; fine-grained matrix encompassing subpellicular microtubules and forming a dense layer below plasmalemma; no specific outer envelope (Fig. 4E).

Development of *B. papi* in the midgut of *Pyrrhocoris apterus*

The flagellates occupied mainly the host's midgut. In firebugs, the midgut is subdivided into four morphologically distinct regions numbered in the antero-posterior direction

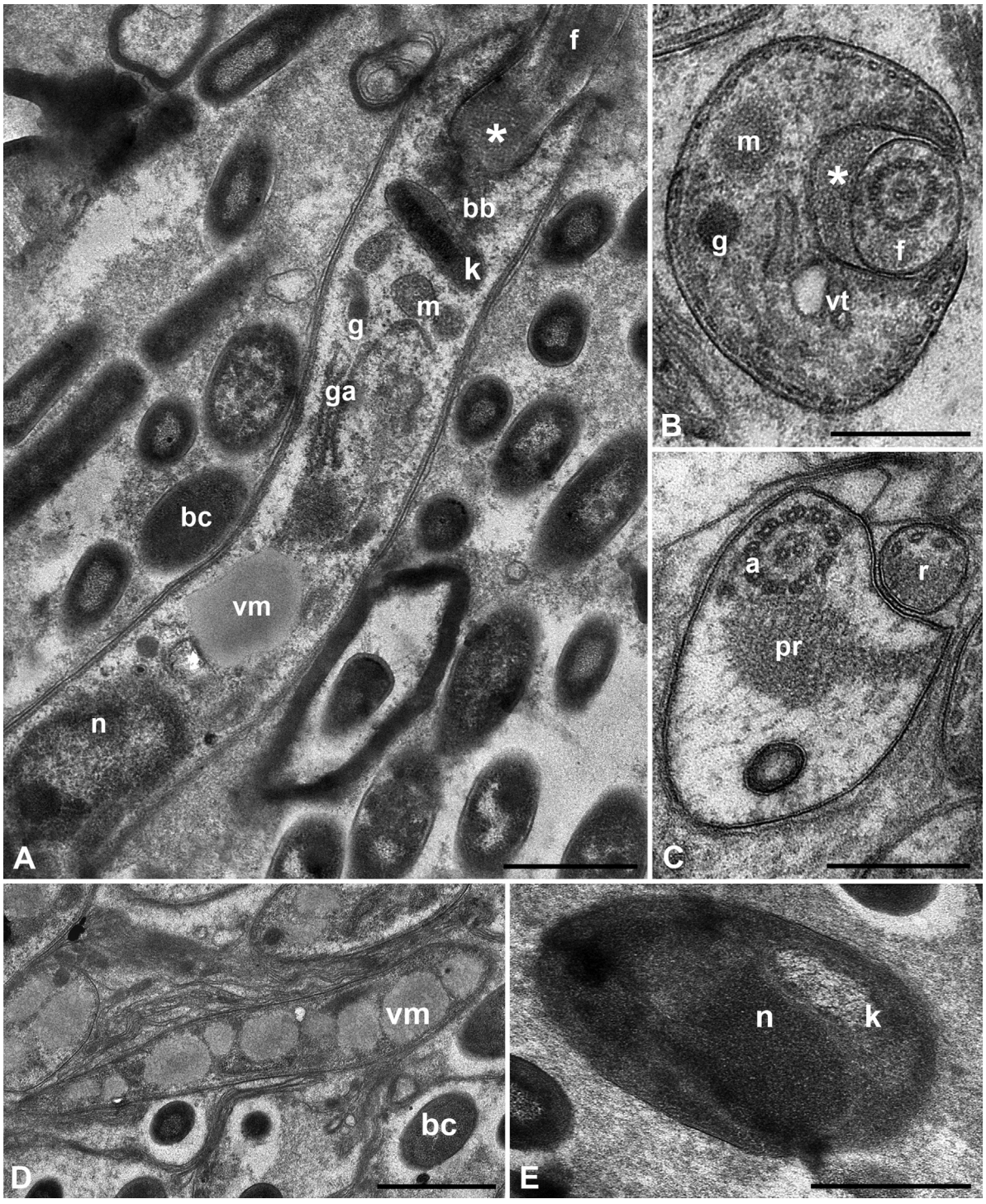

Fig. 4. Ultrastructure of *B. papi* cells in the host midgut. **A** – longitudinal section; **B** – cross section on the level of flagellar pocket filled with dense granular content; **C** – cross section through the apical part of the rostrum, tightly adjoining to the flagellum; **D** – posterior portion filled with vacuoles; **E** – mature "cyst". Asterisk marks granular content of the flagellar pocket. **a** – axoneme; **bc** – bacterial cell; **bb –** basal bodies**; f** – flagellum; **g** – glycosome; **ga** – Golgi apparatus; **k –** kinetoplast; **m** – mitochondrion; **n** – nucleus; **pr** – paraxial rod; **vm –** vacuole with content of medium electron density; **vt** – vacuole with with electron-transparent content;. Scale bars: **A** – 0.8 mkm; **B**, **C** – 0.5 mkm; **D**, **E** – 0.8 mkm.

Table 1. Morphometry of *Blastocrithidia papi* in the midgut of *Pyrrhocoris apterus* and in the xenic laboratory culture.

	Epimastigotes in the midgut N = 31	Epimastigones in culture N = 31	Cyst-like amastigotes in culture N = 31
Cell length (μm)	20.6 ± 5.0 (11.3–31.7)	19.3 ± 3.5 (10.6–25.4)	2.9 ± 0.4 (2.1–3.4)
Cell width (μm)	1.8 ± 0.3 (1.3–2.5)	1.8 ± 0.3 (1.2–2.8)	2.2 ± 0.4 (1.6–2.9)
Nucleus length (μm)	2.0 ± 0.5 (1.2–3.0)	2.1 ± 0.4 (1.4–3.1)	N/A
Nucleus width (μm)	1.0 ± 0.2 (0.5–1.5)	1.1 ± 0.3 (0.5–2.2)	N/A
Anterior end to kinetoplast distance (μm)	6.5 ± 2.7 (2.0–14.2)	6.4 ± 2.1 (2.4–12.8)	N/A
Anterior end to nucleus distance (μm)	8.4 ± 2.9 (3.4–14.4)	7.9 ± 1.9 (3.9–13.4)	N/A
Free flagellum length (μm)	14.7 ± 3.4 (9.0–24.0)	19.7 ± 4.8 (11.8–28.8)	–

as M1–M4 (Haas and Konig 1987). Epimastigotes of *B. papi* were detected in all these sections. However, from M1 to M3 their number was increasing, whereas in M4 (as well as in hindgut) the parasites appeared transiently with fecal masses discharged from M3. Besides, epimastigotes were also observed in the lumen of Malpighian tubules. *P. apterus* individuals in diapause had flagellates concentrated in M3.

The characteristic feature of these bugs' midgut organization is the presence of extracellular membrane layers, ECML (Tieszen and Molyneux 1989) submerged into a jelly-like substance (Frolov and Skarlato 1995). They form a heavy peritrophic structure, isolating chyme from microvilli of enterocytes and are especially abundant in the space between epithelial plicae. Epimastigotes of *B. papi* were detected both in chyme (Figs 4A, 5A, C) and in ECML (Fig. 5B, E). In the M3 segment, chyme contains bug's bacterial symbionts (Haas and Konig 1987), which form aggregates (Fig. 5A). We revealed that the parasites could penetrate such aggregates, though not entering into direct contact with them (Fig. 5A). Numerous epimastigotes formed rosettes uniting cells of similar size with intertwined flagella (Fig. 5A). In the ECML-filled space between intestinal plicae these rosettes were especially abundant and formed "cysts".

Many flagellates and bacteria were also located in capsules formed by concentric membrane layers (Fig. 5A, C). The number of membranes in the walls of such capsules varied from 3 to 20, being altogether up to 100 nm and more thick (Fig. 5C, inset). The flagellates could use ECML as a substrate. For that, discrete hemidesmosomes were formed on the inner surface of parasites' flagellar membrane (Fig. 5D, E). In other cells, flagella penetrated the brush border of enterocytes and anchored there with their swollen distal part (Fig. 5F, G).

Horizontal and vertical transmission of *B. papi* in *P. apterus*

In order to determine the modes of *B. papi* transmission we carried out several experiments (see Section 'Material and Methods' for technical details).

The first experiment was intended to determine presence and way of vertical transmission. We revealed that bugs hatched from eggs with sterilized surface had no parasites, whereas their counterparts from ethanol-untreated eggs were infected (Fig. 6, panel I).

In the second experiment, horizontal transmission via contaminated substrate was tested. Uninfected nymphs were incubated with infected imagines. Gradually all or almost all bugs of the experimental group became infected, while separately kept individuals (control group) remained parasite-free (Fig. 6, panel II).

The third experiment was designed to assess the possibility of horizontal transmission via necrophagy. Analogously to the previous variant, uninfected nymphs were incubated with corpses of infected imagines. The results were similar to those of the second experiment (Fig. 6, panel III).

Discussion

Generic assignment

B. papi in the intestine of its host *P. apterus* is represented by two cell morphotypes: fusiform slender epimastigotes and small specialized amastigotes – "cysts". According to the traditional morphotype-based classification of trypanosomatids (Hoare and Wallace 1966) the new species should be assigned to the genus *Blastocrithidia* Laird, 1959. However, it is noteworthy that the genus in its classical sense was revealed to be polyphyletic (Merzlyak et al. 2001) and one species (*B. culicis*) was already transferred to another genus (Teixeira et al. 2011). Moreover, the phylogenetic position of the type species (*B. gerridis*) is unknown and therefore any taxonomical conclusions concerning the representatives of the genus should be considered temporary.

Phylogenetic affinities of the new species

According to our results *B. papi* nests within the clade of *Blastocrithidia* spp., specifically in the subclade of *B. triatomae*. Biodiversity studies demonstrated that this phylogroup is rich in species, however most of them have not been

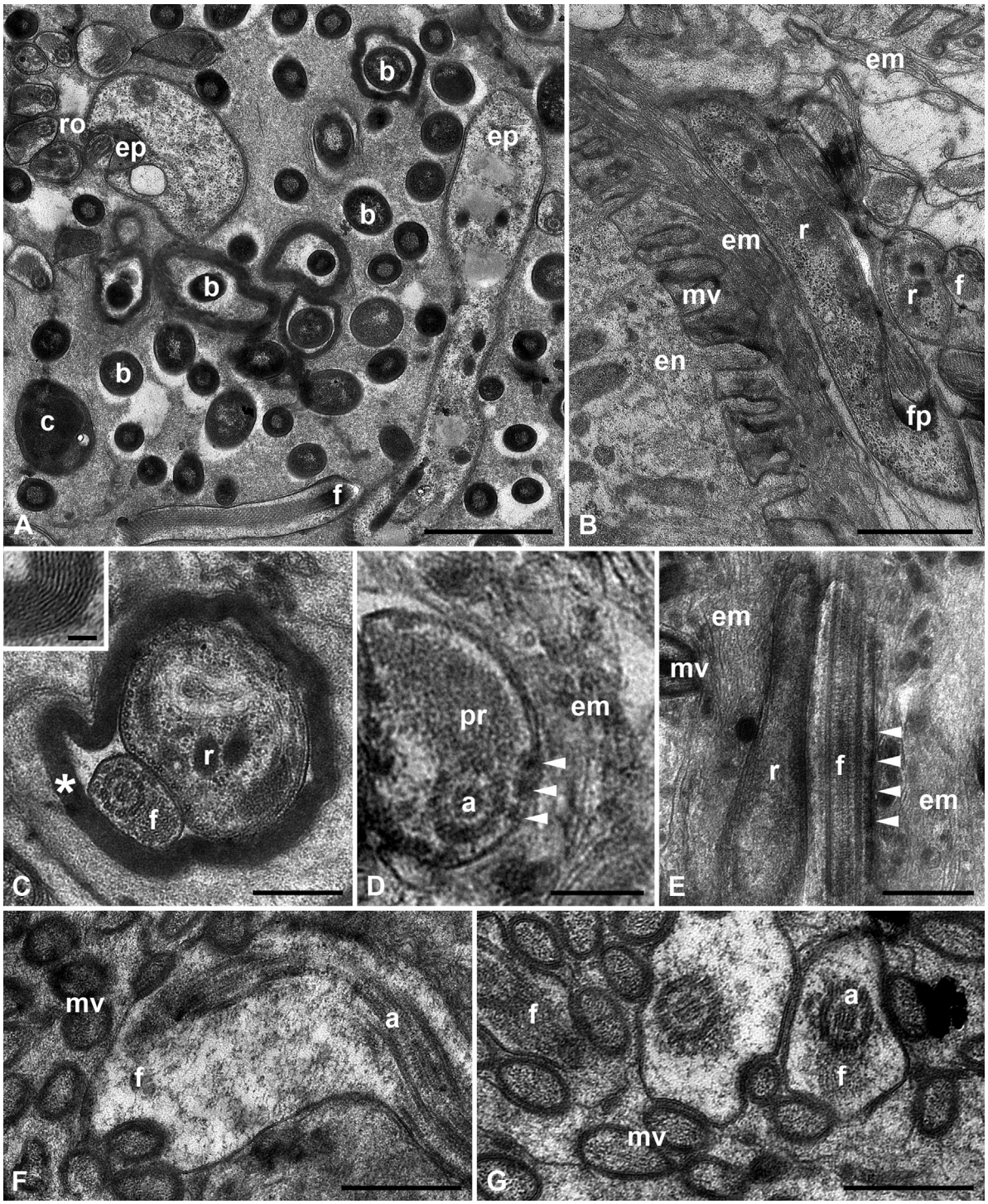

Fig. 5. Development of *B. papi* in the M3 region of midgut of *Pyrrhocoris apterus* (TEM). **A** – cross section through the distal part of the rosette-like cluster of epimastigotes with a mature "cyst" situated nearby, note many bacterial cells either free or enclosed within ECML; **B** – epimastigotes in the lumen between epithelial plicae on the ECML border; **C** – flagellate "encapsulated" within ECML, multiple layers of the "capsule wall" (asterisk) are shown with higher magnification on the inset; **D**, **E** – attachment of epimastigotes to ECML using hemidesmosomes (arrowheads) in transverse and longitudinal sections, respectively; **F**, **G** – anchoring of the swollen distal part of flagellum in the brush border of enterocytes in longitudinal and transverse sections, respectively. **a** – axoneme; **b** – bacterium; **c** – "cyst"; **em** – ECML, **en** – enterocyte; **ep** – epimastigote; **f** – flagellum; **mv** – microvilli; **pr** – paraxial rod; **r** – rostrum; **ro** – rosette of epimastigotes. Scale bars: **A** – 1.5 μm; **B** – 0.9 μm; **C** – 0.4 μm (inset – 50 nm); **D** – 0.2 μm; **E** – 0.5 μm; **F**, **G** – 0.4 μm.

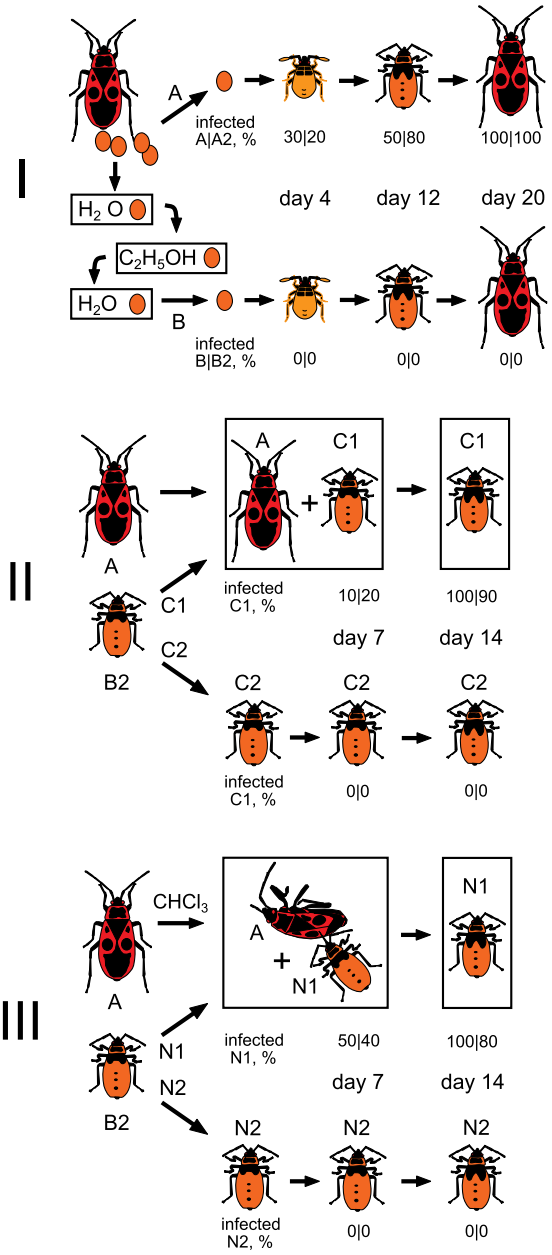

Fig. 6. Experiments on the transmission of *B. papi*. **I** – vertical transmission; **II** – horizontal transmission via contaminated substrate; **II** – horizontal transmission via coprophagy.

yet described or correlated to earlier descriptions (Kozminsky et al. 2015; Votýpka et al. 2010, 2012a).

The closest described relative of *B. papi* is *B. largi*. They are so similar in SSU rRNA gene sequences (>99.9% identity, only two substitutions) that one could consider them one species. However the analysis of the SL RNA gene showed that *B. papi* and *B. largi* are not even sister species. Their differences in the sequences of this gene (20%) are twice as large as the adopted interspecific minimum (Maslov et al. 2007; Westenberger et al. 2004). Both species have closer relatives (TU10, TU11, TU59), which have not yet been described. The small differences in the SSU rRNA gene sequences seem to be typical for the genus (Fig. 1 and Votýpka et al. 2010, 2012a). That may be related either to a slower evolution of this gene (as compared to other trypanosomatids) or to recent diversification of the group.

Morphology of *B. papi*

We revealed in *Blastocrithidia papi* a number of characters, which may be useful for future taxonomic work, such as revision of the genus *Blastocrithidia.* The most significant one is the organization of the anterior end of the cells, the rostrum. Previously it was demonstrated that the corset of subpellicular microtubules at the site of contact with flagellum has a breach in trypanosomes, but is continuous in *Blastocrithidia* spp. (Frolov and Karpov 1995; Taylor and Godfrey 1969). In this aspect, *B. papi* rostrum resembles that of trypanosomes. However, there are also some differences: ridges of endoplasmic reticulum and desmosome contacts are not present in this zone. It is quite likely that this feature is typical of the *B. triatomae* subclade, of which *B. papi* is a member. However, there are no data concerning the ultrastructure of the rostrum in other species of this group.

The presence of electron-dense granular content in the flagellar pocket distinguishes *B. papi* from many other kinetoplastids. The flagellar pocket in trypanosomatids is known to be the main portal for host–parasite interactions (Gull 2003). In particular, several flagellar pocket-associated proteins of African trypanosomes were reported to contribute to trafficking and virulence (Field and Carrington 2009). In all studied representatives of the family, the membrane of this structure participates in endo- and exocytosis (Alcantara et al. 2014; Frolov and Karpov 1995; Gadelha et al. 2009; Overath et al. 1997). Therefore, the lumen of the flagellar pocket may contain excreted material (membrane-enclosed vesicles, single granules, etc.) submerged in a transparent diffuse substance (Martin and Desser 1990). However, in the majority of studied trypanosomatids, the flagellar pocket appears to be empty (Frolov and Karpov 1995). In *B. papi* there is also a relatively large transparent vacuole close to the flagellar pocket. Despite their spatial proximity, no content exchange was detected between the two structures. Thus, the nature and the function of the material filling the flagellar pocket remain unclear.

The presence of a big number of large vacuoles with fine fibrous content in the posterior part of epimastigotes is also a peculiar trait of *B. papi*, which has never been reported in other monoxenous trypanosomatids, including the cyst-forming ones (Caicedo et al. 2011; Frolov and Karpov 1995; Malysheva et al. 2006; Maslov et al. 2010; Podlipaev and Frolov 1987; Romeiro et al. 2000; Schaub and Böker 1986; Tieszen and Molyneux 1989; Tieszen et al. 1985). Nevertheless, in some papers TEM pictures display a concentration of medium-density large vacuoles in the caudal part of the cells, e.c. in long promastigotes of *Herpetomonas trimorpha* (Fig.

1J in Zídková et al. 2010). Therefore, it is possible that this trait is more widely present in trypanosomatids.

Host-parasite relationships and cysts

Monoxenous trypanosomatids parasitizing bugs occupy mostly the lumen of midgut and hindgut (Frolov and Skarlato 1995). Colonization of haemolymph, stomodeal valve and other organs of heteropterans can be encountered only in a few species and apparently are of secondary origin (Frolov 1987; Frolov et al. 2014, 2016a, 2016b; Shaglina et al. 1995). The development of *B. papi* is confined to the M2 and M3 regions of the midgut which are rich in ECML formed as a result of continuous renovation of microvilli (Billingsley and Downe 1983; Frolov and Skarlato 1995; Tieszen and Molyneux 1989; Tieszen et al. 1986). It is interesting to compare the development of *Leptomonas lygaei* and *Blastocrithidia familiaris* from *Lygaeus pandurus* (Lygaeidae) as well as *L. pyrrhocoris* and *B. papi* from *P. apterus*. ECML in the midgut of *Lygaeus pandurus* are distributed unevenly (Tieszen and Molyneux 1989; Tieszen et al. 1986). Promastigotes of *L. lygaei* usually contact with ECML, which may surround single flagellates. These parasites never attach to microvilli even when in close proximity (Tieszen and Molyneux 1989). On the contrary, *B. familiaris* epimastigotes do not contact with ECML at all. Instead they find their breaches and anchor in the brush border of enterocytes seizing microvilli with the swollen flagellar tip (Tieszen et al. 1986). *L. pyrrhocoris* behaves similarly to *L. lygaei* and localizes mainly in the chyme, between and inside ECML (Frolov and Skarlato 1995). *B. papi* epimastigotes form two micropopulations: one in the chyme (similarly to *L.* pyrrhocoris) and another on the inner border of ECML in the vicinity of microvilli. Their swollen flagellar tips wedge between ECML and attach to them using hemidesmosomes. Besides, the flagella of *B. papi* may penetrate into the zone of microvilli and anchor there in the same way as *B. familiaris* and several other trypanosomatids (Frolov and Skarlato 1988; Frolov and Skarlato 1995; Tieszen et al. 1986). Thus, ECML and similar structures may restrict the possibility of attachment to host enterocytes' microvilli by a variety of trypanosomatids, whilst other species find a way to do that.

Transmission of *B. papi* by *P. apterus*

The presence of horizontal and vertical transmission was experimentally confirmed in the life cycles of several monoxenous trypanosomatids of bugs (Carvalho and Deane 1974; Dias Fde et al. 2014; Frolov and Malysheva 1993; Malysheva and Frolov 1995; McGhee and Cosgrove 1980; Schaub and Jensen 1990). Here we demonstrate for the first time a complex life cycle strategy, including the two modes in the population of one host species. Vertical transmission of *B. papi* occurs *transovum* as in cyst-forming trypanosomatids from lygaeid bugs (Dias Fde et al. 2014; McGhee and Cosgrove 1980). This process was described in detail for *Coriobacterium glomerans*, an obligate bacterial symbiont of *P. apterus*, necessary for digesting dry linden seeds (Kaltenpoth et al. 2009). Firebugs developed a special behavioral model in order to ensure acquisition of the bacterium by their progeny. Females contaminate the eggs' surface with their feces, which are engulfed by newly hatched nymphs. *B. papi* obviously exploits this mechanism guaranteeing its effective transmission. The formation of resistant cells (i.e. "cysts") of this trypanosomatid takes place in the intestinal segment inhabited by *C. glomerans*. Both microbes are spatially associated with each other and are transmitted simultaneously.

Besides vertical transmission, we documented two modes of horizontal transmission in *B. papi*: via contaminated substrate and necrophagy. Both are based on the same model of *P. apterus* behavior: they explore different organic substrates all the time (Socha 1993). The efficiency of these two modes is determined by the high density of firebugs both in natural and laboratory colonies.

Prokaryotic symbionts play an important role in the digestive process of many insects (Shigenobu et al. 2000; Zientz et al. 2004). Heteropterans are not an exception and they demonstrate a wide range of modes of vertical transmission of symbionts from parents to progeny (Beard et al. 2002; Fukatsu and Hosokawa 2002; Kaltenpoth et al. 2009). We argue that trypanosomatids, being common parasites of true bugs, may assimilate these mechanisms.

Taxonomic summary

Class Kinetoplastea (Honigberg, 1963) Vickerman, 1976

Subclass Metakinetoplastina Vickerman, 2004

Order Trypanosomatida (Kent, 1880) Hollande, 1952

Family Trypanosomatidae (Doflein, 1901) Grobben, 1905

Genus *Blastocrithidia* Laird, 1959

Blastocrithidia papi sp. n. Frolov and Kostygov, 2016

Species diagnosis: fusiform epimastigotes 11–31 μm long, with tapered ends, of which the anterior one forms a conspicuous rostrum; flagellum length not greater than that of the cell body; rostrum with breached corset of subpellicular microtubules at the site of contact with flagellum; no desmosomes between flagellum and rostrum; flagellar pocket filled with electron-dense granular material; kinetoplast measures 650 × 117 nm; caudal part of epimastigotes filled with big vacuoles of medium electron density; mature "cysts" measure 2.8 × 2.2 μm. Reference sequences are KX641340 (SSU rRNA), KX641366–KX641369 (5S/SL RNA repeat region).
Type host: *Pyrrhocoris apterus* Linnaeus, 1758 (Heteroptera: Pyrrhocoridae).

Site: Midgut (M2 and M3 regions) and Malpighian tubules.
Type locality: Colony near Berezitsy village, Pskov region, Russia (58°35′N; 28°55′E).
Type material: Xenotype (Pap_114_13), hapanotype (cpp_050615_3) and xenic culture (Bl_Pa3) are deposited in the research collection of the laboratory of Protozoology of the Zoological Institute of the Russian Academy of Sciences.
Etymology: Species name of *Blastocrithidia papi* is derived from the abbreviation of its host's binominal name – "P" for *Pyrrhocoris* and "ap" is for *apterus* in genitive case.

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