Parasitol Res (2014) 113:4207–4215
DOI 10.1007/s00436-014-4096-1

ORIGINAL PAPER

Reisolation and redescription of *Balantidium duodeni* Stein, 1867 (Litostomatea, Trichostomatia)

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Received: 20 July 2014 / Accepted: 25 August 2014 / Published online: 4 September 2014

Abstract In this work, we present reisolation and redescription of *Balantidium duodeni* Stein, 1867 from the European common brown frog *Rana temporaria* Linnaeus, 1758 using light and electron microscopy. This species has a unique morphological feature—its cells are flattened along the dorsoventral axis. Because of its unique morphology and localization (duodenum) in the gastrointestinal tract of the host, it has been proposed to recognize *B. duodeni* as a member of separate genus, *Balantidiopsis* Penard, 1922. Molecular phylogenetic analysis demonstrates it to be close to the type species *Balantidium entozoon* (Ehrenberg, 1838). We argue that its placement into separate genus is not substantiated. We also propose to reinstate the genus *Balantioides* Alexeieff, 1931 with the type species *Paramecium coli* (Malmstein, 1857). The recently proposed generic name for this taxon, *Neobalantidium* Pomajbíková et al., 2013, is a junior synonym of the previously recognized name.

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Keywords *Balantidium duodeni* · Phylogeny · 18S rRNA · Transmission electron microscopy

Introduction

The genus *Balantidium* Claparède & Lachmann, 1858 unites over 80 species of ciliate protists, parasitizing intestine of numerous vertebrates and invertebrates (Li et al. 2007, 2009; Lynn 2008; Schuster and Visvesvara 2004). The current taxonomy of this group is based mainly on morphometrical measurements of trophozoites (feeding stage) as other morphological traits cannot be used to delineate species. Yet, the system is contentious because trophozoites can be extremely polymorphic (Esteban et al. 1998; Ponce-Gordo et al. 2008). The diagnostic set of traits usually includes cell-, macro-, and micronuclei sizes; vestibulum length; and numbers of dorsal and ventral kineties. The presence of "Villeneuve-Brachon's" (V-B) field of somatic cilia to the right of the vestibulum or in a dextroral location was considered as another diagnostic feature of this genus (Grim and Buonanno 2009). Many of the old descriptions used only a subset of these parameters and thus can provide very little, if any, information for species identification and comparison. Moreover, some species were described on the basis of a limited number or even single specimens (i.e., *Balantidium gracilis* Bezzenberger, 1904). Provided well-documented variability of ciliates, such descriptions are rendered futile. Comparative analysis of several described *Balantidium* spp. parasitizing fish and amphibians indicated that many of these specific names may be synonyms (Kornilova et al. 2014). The only possible solution to overcome this hurdle is reisolation and formal redescription of as many previously characterized species as possible. This should be done using modern electron microscopic techniques and molecular phylogenetic approaches. To date, only few *Balantidium* spp. were scrutinized in this way. Understandably, most of the

research attention has been focused on medically important parasites. *Balantidium coli* (Malmstein, 1857) is a broadly distributed ciliate inhabiting the intestine of many mammalian hosts and causing balantidiasis. Domestic pigs are considered as principal hosts and a major reservoir of this parasite (Schuster and Ramirez-Avila 2008). Based on molecular similarities of their 18S and ITS1-5.8S-ITS2 sequences, many different strains isolated from mammals and birds were recently united into just one species—*B. coli* (Ponce-Gordo et al. 2008, 2011). Despite their morphological similarities, these cyst-forming ciliates are phylogenetically distant from the type species of the genus *Balantidium*, *Balantidium entozoon* (Ehrenberg, 1838). This served as a basis for its separation into a distinct genus *Neobalantidium* (Pomajbíková et al. 2013).

The vast majority of balantidia from other host groups (fishes, amphibians, and reptilia) were described using light microscopy. Only some of *Balantidium* species from this set were reanalyzed in the modern times using scanning or transmission electron microscopy (SEM and TEM, respectively). These are fish parasites *B. jocularum* Grim, 1993, *B. polyvacuolum* Li, 1963, *Balantidium ctenopharyngodonis* Chen, 1955, *B. macrodextroral* Grim et al., 2002 (Grim 2006; Grim et al. 2002; Li et al. 2007, 2009), and amphibian parasites *B. entozoon*, *B. xenopi* de Puytorac and Grain, 1965, and *B. honghuensis* Li, 2013 (de Puytorac and Grain 1965; Grim and Buonanno 2009; Guinea et al. 1992; Li et al. 2013). Even less is known about phylogenetic relationships between different balantidia. Analysis of the 18S ribosomal RNA sequences of the type species *B. entozoon* isolated from *Pelophylax* kl. *esculentus* (Linnaeus, 1758) and *B. ctenopharyngodonis* from the hindgut of grass carp *Ctenopharyngodon idella* (Valenciennes, 1844) demonstrated that these two species might be phylogenetically separated (Li et al. 2011).

Balantidium duodeni Stein, 1867 was described from *Pelophylax* sp. collected in the vicinity of Prague, Czechia. It can be distinguished from other balantidia by its unique morphology—cells are flattened along the dorsoventral axis. This feature allows accurate species identification even at the level of light microscopy. It has been proposed to place it into a distinct genus, *Balantidiopsis* Penard, 1922 (Jankowski 2007). In this work, we present reisolation and redescription of *B. duodeni* from the European common brown frog *Rana temporaria* Linnaeus, 1758.

Material and methods

Filed work and light microscopy

Fifteen specimens of the frog host, *R. temporaria* collected in the vicinity of the Vyritsa, Leningrad Region, Russia (59° 24′ 54″ N; 30° 16′ 36″ E) during April and May 2013, were assayed for the presence of *B. duodeni*. All frogs were dissected and the luminal content of their duodenum was collected, washed in 0.6 % NaCl, fixed in 4 % formaldehyde, and examined using Leica DM2500 microscope (Leica Microsystems, Vienna, Austria) equipped with Nikon DS-Fi1 visualization system (Nikon Precision Europe GmbH, Langen (Hessen), Germany).

Transmission electron microscopy

B. duodeni specimens collected for TEM study were washed and fixed in 0.1 M cacodylate-buffered (pH 7.4) 1 % osmium tetroxide and 4 % (*v/v*) glutaraldehyde for 1 h at +4 °C. After extensive washing, cells were postfixed in 0.1 M cacodylate-buffered (pH 7.4) 1 % OsO_4 for 15 min, dehydrated stepwise with increasing concentrations of ethanol, washed once with propylenoxide, and flat embedded in Epon-Araldite resin mixture. Ultrathin sections (70 nm) were cut using a Reichert-Jung Ultracut E ultramicrotome (Leica Microsystems), collected on copper grids, which were contrasted in ethanolic uranyl acetate and lead citrate, and observed in a Tesla BS500 microscope (Tesla, Brno, Czech Republic).

DNA isolation and polymerase chain reaction

DNA was isolated using SDS-proteinase K lysis and salt extraction as described previously (Kostygov and Frolov 2007). The 18S ribosomal RNA (rRNA) gene of *B. duodeni* was amplified using the universal eukaryotic primers A and B (Medlin et al. 1988). The amplification cycle involved an initial denaturation for 2 min at 94 °C, followed by 30 cycles each of 25 s denaturation at 94 °C, 30 s of annealing at 55 °C, and extension at 72 °C for 2 min, followed by 10 min incubation at 72 °C. The PCR product was purified using GFX PCR DNA and Gel Band Purification kit (GE Healthcare Europe GmbH, Freiburg, Germany) and then sequenced directly as described elsewhere (Milyutina et al. 2001). The sequence has been submitted to the GenBank under accession number KM057846.

Phylogenetic analyses

18S rRNA sequences were aligned using Muscle 3.8.3.1 (Edgar 2004) and then the resulting alignment was refined manually using the BioEdit sequence alignment editor (Hall 1999) and subjected to processing with Gblocks (Castresana 2000) using the following parameters: "Minimum length of a block"=5 and "Allowed gap positions"="With half." Other settings were set by default. The resulting alignment of 70 taxa contained 1,539 nucleotide positions. Evolutionary model (GTR+I+G) for this dataset was selected using Akaike criterion in jModeltest 2.1.4 (Darriba et al. 2012) and used for

maximum likelihood phylogenetic inference in RAxML 8.0 (Stamatakis 2014). Heuristic search was performed using TBR branch swapping algorithm. Statistical support of bipartitions was assessed with the use of bootstrap resampling (1,000 "thorough" replicas). Bayesian inference of phylogeny was accomplished in MrBayes 3.2.2 with analysis run for five million generations under GTR+I+ G model (five gamma categories) and sampling every 1,000 generation. Other parameters were left in their default states.

Results

Infection

Out of 15 *R. temporaria* frogs examined, 3 were found to be infected with *B. duodeni* (prevalence, 20 %). Number of active trophozoites was estimated at several hundred cells per host specimen. Parasites were mainly found in the proximal part of the small intestine.

Light microscopy

Cells were from 111.6 to 156.9 μm ($\bar{x}$=128.6±3.0; hereafter n=20) in length and 86.1 to 123.3 μm (101.4±2.7) in width. The average ratio of length to width was between 1.18 and 1.32 (1.27±0.01) (Fig. 1a, b). Cell body was noticeably flattened. The ventral side was concaved. A small notch could be noticed on the anterodorsal side of the cell (Fig. 1c). Uniform rows of somatic kineties situated about 0.7 μm apart. The total number of kineties reached 360–380 at the cell equator. The average length of the cilia was 7.1 μm. The vestibulum measured between 44.2 and 76.7 μm (56.3±2.5) and was located in the anterior part of the cell (Fig. 1a, d). Its edges were covered by shorter vestibular kineties angled at about 135° to their somatic counterparts (Fig. 1d). These shorter kineties were also situated about 0.7 μm apart. The ratio of the vestibulum length to that of the cell body was between 0.4 and 0.6 (0.44±0.01). Oval macronucleus was usually found in the posterior part of the cell and measured between 37.2 and 48.8 μm (41.8±0.7) in length and 23.3 to 41.9 μm (30.0±1.3) in width. The micronucleus was always found in a close proximity to the macronucleus. The contractile vacuole located in the posterior part of the cell not far from the nuclei.

Electron microscopy

Rows of evenly spaced somatic kineties on the cell surface were separated by interkinetal ridges that measured between 700 and 900 nm (Fig. 2a). Correspondent ridges between vestibular kineties were 200–300 nm wide (Fig. 2b). Cell coverings were represented by the typical pellicula, but the layer of alveoli was not pronounced. Sub-alveolar pellicular microtubules could be detected in some sections. As in other Trichostomatia, the monokinetid of *B. duodeni* was composed of a cilium, a single kinetosome, and fibrillar structures (Fig. 2b). A dense microfibrillar layer (100–200-nm thick, the so called *lamina corticalis*) separated the ectoplasmic and endoplasmic regions (Fig. 2a, b). Numerous electron-transparent vacuoles could be detected just beneath this structure (Fig. 2c). A well-developed system of nemadesms was noticeable in the cytoplasm, especially in the posterior end of the cell (Fig. 2c). Multiple round or oval electron-dense bodies with a diameter of 200 to 400 nm were conspicuous around nemadesmal complexes (Fig. 2c). In most cases, cytoplasm appeared to be partitioned into two different regions (dorsal and ventral). There were numerous microfilaments oriented along the central axis of the cell located at the boundary between these two zones (Fig. 2d). The cytoplasm of the dorsal side of the cell contained numerous electron-dense bodies and associates of microtubules as well as multiple fusiform vesicles (presumably, hydrogenosomes). As for the ventral side, the cytoskeletal elements were less pronounced, fusiform vesicles were absent, and many oval-shaped bacteria were enclosed within individual vacuoles (Fig. 2d).

Phylogenetic analysis

The trees inferred using both maximum likelihood and Bayesian approaches demonstrated that phylogenetic signal-to-noise ratio was comparatively low. This could be easily deduced from the fact that many branches had no statistically significant support (especially conspicuous in bootstrap values, Fig. 3). Moreover, there was also a substantial heterogeneity of branch lengths. The tree topologies were similar to those published previously (Ito et al. 2014; Pomajbíková et al. 2013).

Species of the family Balantidiidae formed three separate clusters. The type species of the genus *Balantidium*, *B. entozoon*, grouped together with *B. duodeni* and uncharacterized *Balantidium* sp. isolate F7. Based on phylogenetic distances, this isolate is different from *B. entozoon* and *B. duodeni*. The position of the clade was unstable as judged from the low statistical support. The second group consisted of several isolates of *B. coli*. Interestingly, some of these isolates appeared as far apart from each other as different species. The third group was represented by a single species *B. ctenopharyngodonis*. It appeared as a sister group of the family Amylovoracidae. This position was strongly supported by both bootstrap value and posterior probability.

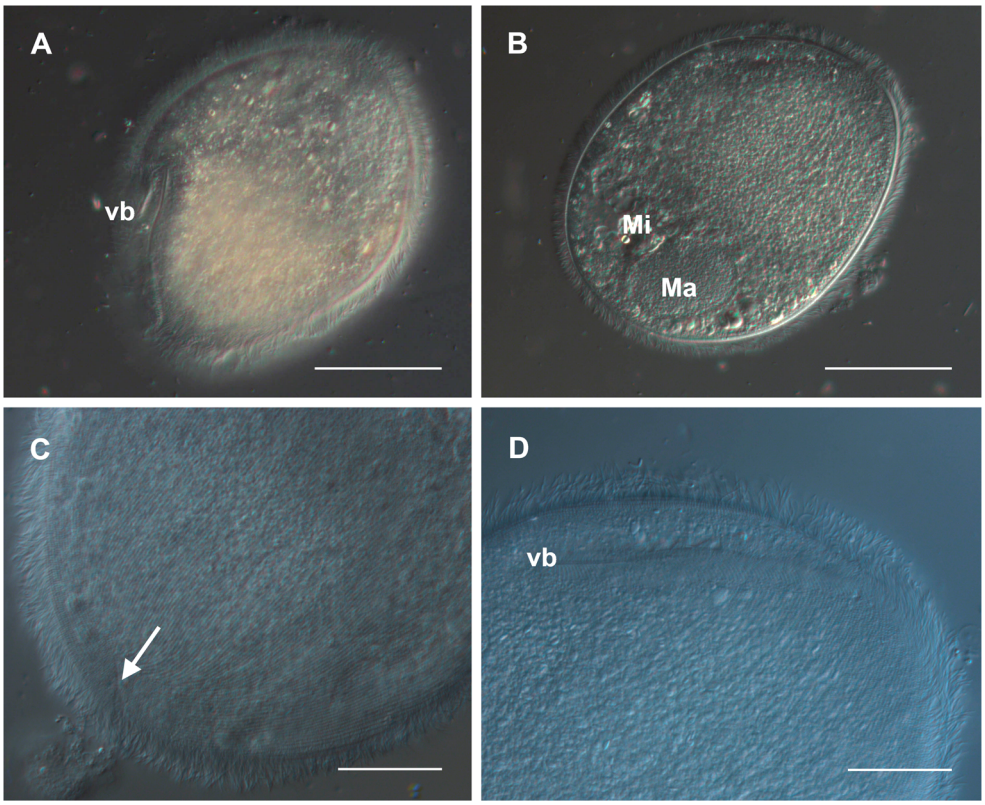

Fig. 1 Morphology of *B. duodeni* by light microscopy. **a**, **b** general morphology, **c** details of the anterodorsal side of the cell, and **d** structure of the vestibulum. *Arrow* in **c** denotes a notch. *vb* estibulum, *Ma* macronucleus, *Mi* micronucleus

Discussion

According to Stein's report (Stein 1867), *B. duodeni* was found only in green frogs that he referred to as *Rana esculenta* (Linnaeus, 1758). We believe that the author did not distinguish the hybrid species *Pelophylax* kl. *esculentus* and its both parent species: marsh frog *Pelophylax ridibundus* (Pallas, 1771) and pool frog *Pelophylax lessonae* (Camerano, 1882), especially given that the last one was not described at that time. Our view is justified by the results of

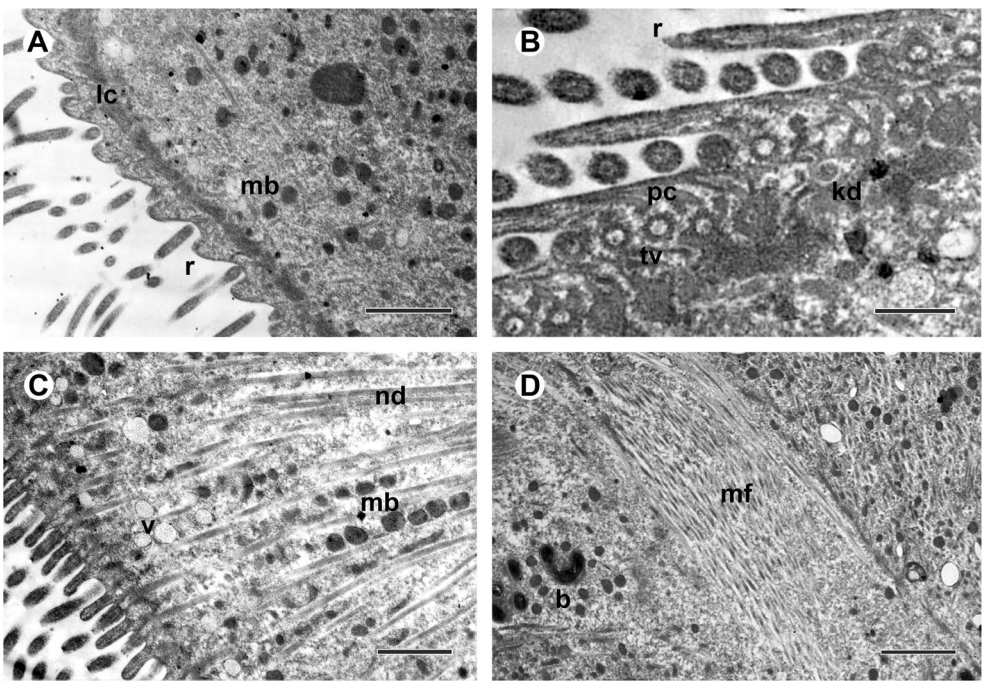

Fig. 2 Ultrastructure of *B. duodeni*. **a** somatic cortex, **b** cortex in the vestibulum zone, **c** posterior part of the cell, and **d** microfilaments in the central axis of the cell. *lc lamina corticalis*, *r* cortical ridges, *mb* microbodies, *tv* transversal microtubules, *pc* postciliary microtubules, *kd* kinetodesmal filament, *v* vacuoles, *nd* nemadesm, *mf* microfilaments, *b* bacteria

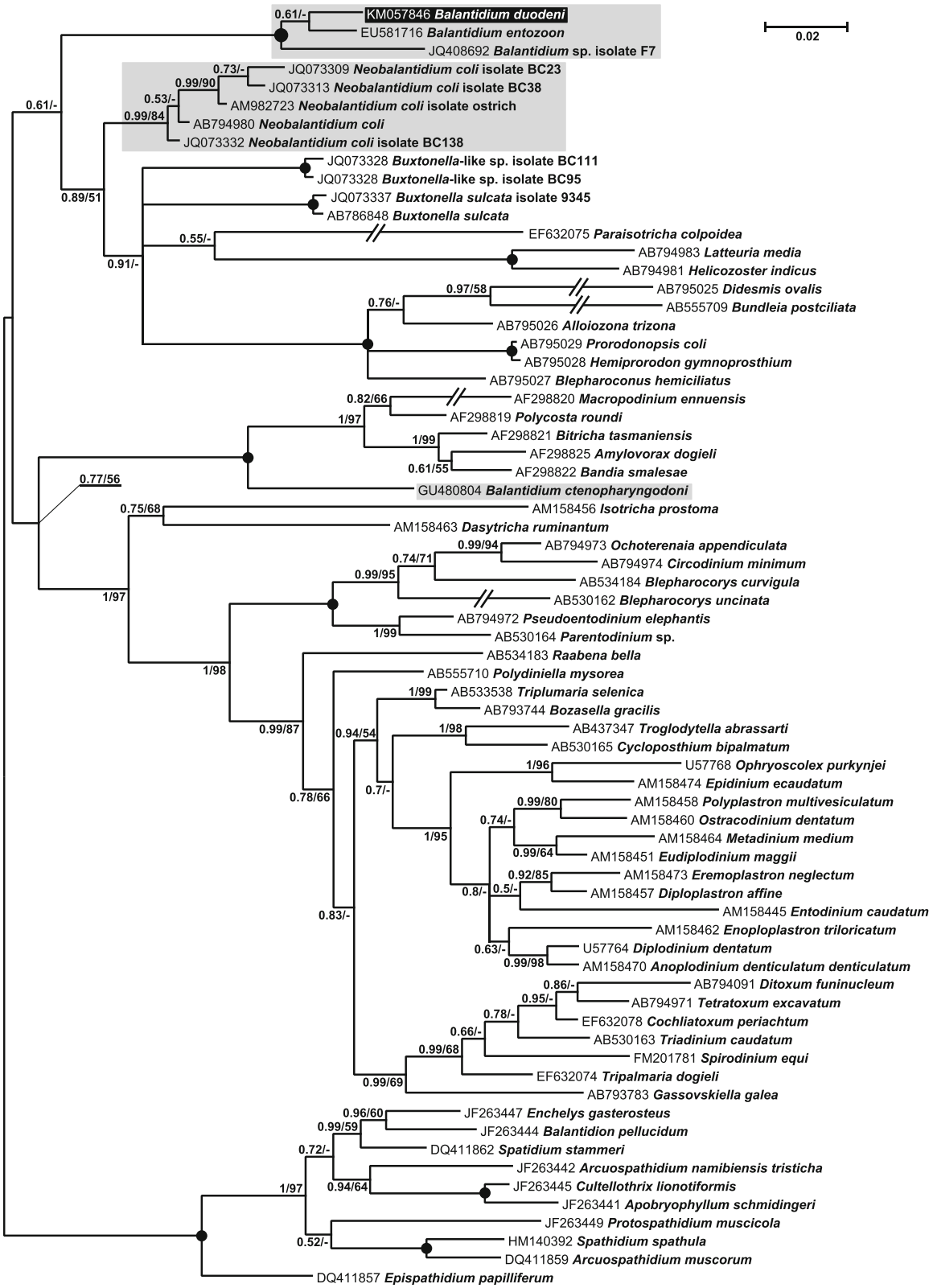

Fig. 3 Phylogenetic tree showing the position of *B. duodeni* inferred by maximum likelihood method using 18S rRNA set. The length of all branches is proportional to the number of substitutions per site. Double-crossed branches are at 50 % of their original lengths. Numbers at nodes indicate posterior probability and bootstrap percentage, correspondingly. Values less than 0.5 and 50 % are replaced with dashes. Nodes having 1.0 posterior probability and 100 % bootstrap support are marked with black circles. The *bar* represents number of substitutions per site. *B. duodeni* and other species of *Balantidium* and *Neobalantidium* (*Balantioides*) are highlighted

subsequent studies that recorded the presence of *B. duodeni* in all these host species as well as in common frog *R. temporaria*, dark-spotted frog *Pelophylax nigromaculatus* (Hallowell, 1861), and Indian bullfrog *Hoplobatrachus tigerinus* (Daudin, 1803) (Table 1) (Nie 1935; Senler and Yildiz 2000; Sukhanova 1960). The host specificity of balantidia of cold-blooded vertebrates is still insufficiently investigated. Nevertheless, there is evidence that the spectrum of amphibian hosts of *B. entozoon*, *B. duodeni*, and *Balantidium elongatum* is rather wide (Kornilova et al. 2014; Sukhanova 1960).

Unlike other endosymbiont ciliates, *B. duodeni* was being found mainly in the duodenum and stomach of frogs, with only a few cells revealed in the rectum. Original description of the species did not contain any information about cysts, but they were reported in later studies (Sukhanova 1960). In our material collected in spring, we observed trophozoites only. This correlates with the published data on the life cycle of *B. duodeni*: cystic and precystic stages are formed in late summer and early autumn.

The representatives of the genus *Balantidium* have several common morphological features. Their cell body is sacciform or slightly elongated and is completely covered with cilia forming dense longitudinal rows. The lateral edges of the peristome of these protists are bordered with two bands of even closer arranged cilia that are different from somatic kineties (Lynn 2008). Such arrangement is clearly visible on micro-photos (Fig. 1b, d). Because balantidia have very few other morphological characters suitable for taxonomy, their species identity is usually determined using morphometric data. Main cell measurements of *B. duodeni* in our study proved to be quite similar to those given in the original description, though somewhat different from the data published by other authors (Table 1). We suppose that some authors may have analyzed different species morphologically similar to *Balantidium duodenum* (Bhatia 1936; Nie 1935). Such situation is rather common in parasitology and protistology. Several prominent examples can be found in our work on Trypanosomatidae (Maslov et al. 2013; Votýpka et al. 2010).

Table 1 Comparison of cell size ranges of *B. duodeni* according to different authors

Reference	Length, μm	Width, μm	Host species and collection locality
Current study	112–157	86–123	*Rana temporaria* Leningrad Region
Stein (1867)	104–136	80–115	*Pelophylax* kl. *esculentus* Czechia
Nie (1935)	45–79	36–66	*Pelophylax nigromaculatus* China
Bhatia (1936)	74	56	*Hoplobatrachus tigerinus* India
Sukhanova (1960)	109–172	66–122	*Rana temporaria*, *Pelophylax* kl. *esculentus* *Pelophylax ridibundus* Leningrad Region
(Senler and Yildiz 2000)	55–150	50–88	*Pelophylax ridibundus* Turkey

According to the previously published report, precystic forms of *B. duodeni* are small in size and have almost round shape and hardly discernible peristome. These cells are 60–110-μm long and 56–110-μm wide. The formation of precystic forms of this species in pool frogs and marsh frogs occurs during the whole spring-to-autumn period, but in common frogs, this process takes place only in fall. This distinction arises from the differences in biology of host species. Moreover, balantidia from *P. lessonae* and *P. ridibundus* are smaller than those of *R. temporaria* (Sukhanova 1960). Thus, the discordance in morphometric data published by different authors can also be explained by dissimilarities in physiological condition of parasites inhabiting various hosts and observed in different phases of their life cycles.

In our opinion, the dorsoventral oblateness of cells is an important distinctive character of *B. duodeni*. All other species of ciliates that we found in frogs were circular in cross section. Concave ventral side of the cell functions as a sucker fixing the body of *B. duodeni* on the wall of a frog's intestine. Some authors believed this peculiarity to be substantial for placing this species in the separate genus *Balantidiopsis* Bütschlii, 1889 (Jankowski 2007).

The ultrastructure of *B. duodeni* is quite similar to that of other representatives of the genus *Balantidium* studied to date (Guinea et al. 1992; Li et al. 2007; Nilles-Bije and Rivera 2010). Rows of cilia alternate with evenly distributed cortical ridges. The latter contain elements of ciliary root apparatus and pellicular microtubules. The system of alveoli is poorly developed. *B. entozoon* has a zone of shortened cilia to the right of vestibulum (V-B field) that was proposed as a diagnostic feature of the genus *Balantidium* (Grim and Buonanno 2009). However, we did not found such structure in *B. duodeni*. Moreover, it should be mentioned that V-B field was not revealed in several other *Balantidium* spp. (Li et al. 2007). At the same time, the aggregation of shortened cilia was found not only in various species of the order Trichostomatia but also in other orders within Litostomatea. This encouraged some authors to use this character in the diagnosis of the whole class (Vd'ačný et al. 2011). Cortical ridges of *Neobalantidium coli* contain multiple mucocysts that some authors associate with the ability of these ciliates to encystation (Pomajbíková et al. 2013). In *B. duodeni*, we found no extrusomes though there are reports that these protists also form cysts (see above).

The layer of microfilaments located on the border of ecto- and endoplasm (*lamina corticalis*) in *B. duodeni* is more pronounced than in other representatives of the genus

Balantidium studied to date by electron microscopy. It is probably due to the compressed cell shape of this ciliates and their peculiarity of locomotion. We suppose that well-developed system of nemadesms and thick aggregation of microfilaments along the central cell axis are related to the formation of the sucker on the ventral surface of *B. duodeni*. Probably for the same reason the cytoplasm is differentiated into two zones (denser dorsal with developed cytoskeleton and multiple hydrogenosomes and looser ventral). Such cell structure seems to be unique for *B. duodeni*. Electron-clear vacuoles situated under the layer of cortical filaments were also found in *B. ctenopharyngodonis*, *B. polyvacuolum*, and *B. jocularum*. They are presumed to serve as a depot of Ca^{2+} ions and thus to participate in the regulation of locomotion and cell shape change (Grim 1993).

Membrane-bound electron-dense bodies in the cytoplasm of balantidia by all appearances are hydrogenosomes. These organelles were found in representatives of various groups of endobiontic as well as free-living ciliates inhabiting anaerobic biotopes. The hydrogenosomes were recorded in *B. entozoon* and *B. coli* (the latter was also reported to have mitochondria) (Grim and Buonanno 2009; Lynn 2008; Nilles-Bije and Rivera 2010). Moreover, some electron-dense bodies could be seen on the electron microscopic photos of *Balantidium jocularum* and *Balantidium caviae* (Grim 1993; Paulin and Krascheninnikow 1973). As evidenced by their intracellular location, bacteria in the cytoplasm of *B. duodeni* appear to be symbionts.

According to the molecular phylogenetic analysis, *B. duodeni* and the type species of the genus, *B. entozoon*, proved to be close species and undoubtedly members of the same genus (Fig. 3). Interestingly, another isolate of *Balantidium* sp. (F7) clusters together with abovementioned species. In some previously published papers, this isolate was referred to as *B. entozoon* (isolate F7) (Pomajbíková et al. 2013), but our data testify that this is probably another closely related species of this genus. Because of its unique morphology and localization (duodenum) in the gastrointestinal tract of the host, it has been proposed to recognize *B. duodeni* as a member of separate genus, *Balantidiopsis* Penard, 1922 (Jankowski 2007). Our data argue that such taxonomical revision was not substantiated.

Although the number of *Balantidium* spp. analyzed by molecular methods is low, some preliminary conclusions can be reached. The genus is obviously polyphyletic and splits into three distinct groups according to the host specificity: parasites of fish (*B. ctenopharyngodonis*), amphibia (*B. entozoon*, *B. duodeni*, and *Balantidium* sp. isolate F7), and warm-blooded vertebrates (*B.* coli). The last one may represent a group of several closely related species. The distinct position of *B. coli* allowed (Pomajbíková et al. 2013) to erect a new genus, *Neobalantidium*. However, we found that this exact taxon has been already named a long time ago as *Balantioides* Alexeieff, 1931. We argue that this name should be reinstated.

Taxonomic summary

Class Litostomatea Small & Lynn, 1981

Subclass Trichostomatia Bütschli, 1889

Order Vestibuliferida de Puytorac et al., 1974

Family Balantidiidae Reichenow in Doflein & Reichenow, 1929

Genus *Balantidium* Claparède & Lachmann, 1858

Balantidium duodeni Stein, 1867

Species diagnosis: *B. duodeni* is a ciliated binucleate protozoan with macro- and micronuclei covered by uniform rows of monokinetid somatic ciliation, an anteroventral oral cavity depressed below the surface, and a vestibular groove leading into the oral apparatus. Cells of *B. duodeni* are between 104 to 172 μm and 66 to 123 μm in length and width, respectively. Cell body is noticeably flattened as compared to other *Balantidium* spp. The ventral side is concaved. A small notch can be noticed on the anterodorsal side of the cell. The vestibulum is located in the anterior part of the cell. Oval macronucleus is usually found in the posterior part of the cell and measures between 37 and 49 μm in length and 23 to 42 μm in width. The species can be identified by the unique 18S rRNA sequence (GenBank accession numbers KM057846).

Type host: *Pelophylax* kl. *esculentus* (Linnaeus, 1758)

Site: duodenum part of the small intestine.

Type locality: vicinity of Prague (50° 03′ 64.7″ N; 14° 22′ 51.5″ E), modern Czech Republic.

Comment: *B. duodenum* has been also isolated from *Rana temporaria* Linneaus, 1758, *Pelophylax nigromaculatus* (Hallowell, 1861), *Hoplobatrachus tigerinus* (Daudin, 1803), and *P. ridibundus* (Pallas, 1771).

Genus *Balantioides* Alexeieff, 1931

Type species: *Paramecium coli* (Malmstein, 1857)

Comment: recently proposed generic name *Neobalantidium* Pomajbíková et al. 2013 is a junior synonym of the previously recognized name *Balantioides* Alexeieff, 1931 (Alexeieff 1931). In accordance with The International Code of Zoological Nomenclature, the name *Balantioides* should be used in this instance.

Acknowledgments This work was done with a financial support of the Russian Foundation for Basic Research (12-04-00767-a) using equipment and resources of the Life Science Research Centre (University of Ostrava), Facility Centers "Culturing collections of microorganisms" and "Chromas" (St. Petersburg State University).

Conflict of interest The authors declare no conflicts of interest.

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