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European Journal of Protistology 45 (2009) 193–204
European Journal of PROTISTOLOGY
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Morphological and molecular characterization of *Histiobalantium natans viridis* Kahl, 1931 (Ciliophora, Scuticociliatia)

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Received 8 August 2008; received in revised form 6 November 2008; accepted 8 November 2008

Abstract

We investigated a *Histiobalantium natans viridis* population from the ancient Lake Biwa in Japan, using live observation, silver impregnation, and the small subunit rRNA gene sequence. The morphological and molecular data show, with high support, a close relationship of *Histiobalantium*, *Schizocalyptra* and *Pleuronema*, supporting the family Pleuronematidae Kent, whose nearest relatives are the Cyclidiidae Ehrenberg. A family Histiobalantiidae Puytorac and Corliss is not supported, either by the nucleotide sequences or the morphologic data, except for the curious dorsal location of the cytopyge. Likewise, the data do not support inclusion of *Histiobalantium* in the family Sulcigeridae Gajewskaja, as very recently suggested by Jankowski, whose classification is based on a misidentified *Sulcigera comosa*. Further, there are good reasons to synonymize the genus *Gajewskiella* Obolkina with *Histiobalantium*. The European and Asian populations of *H. natans* differ significantly in the structure of adoral membranelle 1, suggesting that they are different species. However, there is some indication that the differences are caused by deficient data on the European *H. natans*, which is thus in need of detailed redescription. This applies also to the North American *Histiobalantium agile* Stokes which is probably not conspecific with the Eurasian *H. natans*. The status of the forma *H. natans viridis* and *H. natans nigricans* is not known because detailed data from *nigricans* are lacking. We prefer subspecific rank at the present state of knowledge.

Keywords: Biogeography; *Gajewskiella*; Histiobalantiidae; ICZN; Lake Biwa; Sulcigeridae

Introduction

Histiobalantium Stokes, 1886 comprises middle-sized (40–200 µm) oligohymenophorean ciliates with a large, boat-shaped buccal cavity, the anterior third of which contains three adoral membranelles in a V-shaped pattern. The paroral membrane is 6-shaped and has a characteristic notch in the posterior third, i.e., before it turns around the buccal vertex. Presently, six species are recognized from limnetic and marine ecosystems as well as from benthic and planktonic habitats: *H. natans* (Claparède and Lachmann, 1859) Kahl, 1931 (limnetic; includes the junior synonym *Histiobalantium agile* Stokes, 1886; redescribed by Kahl 1931 and Dragesco and Iftode 1972); *H. majus* Kahl, 1931 (limnetic; redescribed by Dragesco 1968; Grolière 1973; Obolkina

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doi:10.1016/j.ejop.2008.11.003

1991); *H. marinum* Kahl, 1933 (marine and brackish; redescribed by Agamaliev 1972 and Wilbert 1986); *H. semisetatum* Noland, 1937 (marine and brackish; redescription not available); *H. minor* Wilbert, 1986 (limnetic); and *H. bodamicum* Krainer and Müller, 1995 (limnoplanktonic; redescribed with SEM micrographs by Foissner et al. 1999; referred to a new subgenus, *Linostomella*, by Jankowski 2007; for nomenclature, see Aescht 2001). Further, Kahl (1931) split *H. natans* into a forma *viridis* and a forma *nigricans* (for details, see Discussion).

Except for *H. semisetatum*, which might belong to the genus *Pleuronema*, all species have been described or redescribed from silver-impregnated specimens. Thus, the genus is well known, while details on species are at variance. For instance, the structure of adoral membranelle 1 is markedly different in several investigations, i.e., consists of two or three ciliary rows and possesses or lacks a segment at anterior end (Dragesco 1968; Dragesco and Iftode 1972; Grolière 1973; Obolkina 1991; Wilbert 1986). Whether these and other differences are species-specific or caused by insufficient data, remains to be investigated. Likewise, ontogenesis and classification are at variance, mainly because Dragesco and Iftode (1972) could not find a typical scutica, thus supposing a closer relationship of *Histiobalantium* to the Peniculia than the Scuticociliatia. However, later Grolière (1973) observed the scutica in *H. majus*. Accordingly, *Histiobalantium* is now widely considered to belong to the Scuticociliatida, where it represents a monotypic family (Corliss 1979; de Puytorac, 1994; Lynn and Small 2002) which, however, has been questioned by Jankowski (2007), who assigns *Histiobalantium* to the family Sulcigeridae Gajewskaja, 1933.

Against this background, a detailed morphological and molecular investigation of the type species, *H. natans*, appears indispensable. Additionally, the different family classifications need to be discussed because they could be based on misidentified genera and species.

Materials and methods

Materials

Histiobalantium natans viridis was discovered in a mud sample taken manually from the flat shore of Lake Biwa at the end of November 2006. The site was very near to the Lake Biwa Museum and contained various filamentous algae and decaying water plants, especially *Nelumbo nucifera*. See Rossiter (2000) for a detailed description of the lake. *Histiobalantium natans viridis*, which was moderately abundant above and in the mud, could not be found in two plankton samples taken with a fine-meshed net from the north and south basin of the lake. In the environmental sample, *Histiobalantium natans viridis* fed on algae, such as *Trachelomonas* and dinoflagellates. In the laboratory, *Histiobalantium natans viridis* could be cultivated on Eau de Volvic enriched with some squashed wheat grains and a few ml of natural mud. Here, it engulfed mainly bacteria and possibly also heterotrophic flagellates, but declined after some weeks.

Morphological methods

Specimens from the environmental sample and the raw cultures were used for the investigations. Living cells were studied using a high-power oil immersion objective and differential interference contrast. Various silver impregnation methods were used to reveal the ciliary pattern and cytological details. All these methods are described in Foissner (1991). Counts and measurements on silvered specimens were performed at a magnification of $\times 1000$. In vivo measurements were conducted at magnifications of $\times 100$–1000. Illustrations of live specimens were based on free-hand sketches and micrographs; those of impregnated cells were made with a drawing device. Terminology follows mainly Corliss (1979).

Molecular methods

Single live cells of *Histiobalantium natans viridis* were isolated under a light microscope and transferred to sterile Milli-Q water droplets two times to facilitate the removal of contaminants, suspended in 2 µl of sterile Milli-Q water, and placed in 0.2 ml thin-walled PCR tubes. Samples were then frozen at −20 °C until analysis.

PCR amplification. The first round of PCR amplification was carried out on single cells, using two sets of external primers, SR1 and SR12 (Nakayama et al. 1996) and the PCR protocol of Puitika et al. (2007).

Cloning, sequencing, and tree construction. PCR products were purified with Wizard® SV Gel and a PCR Clean-Up System (Promega). Sequencing of two *Histiobalantium natans viridis* specimens was performed on an ABI PRISM® 310 Genetic Analyzer for both DNA strands, using the primers SR1, SR3, SR6, and SR8–SR12 of Nakayama et al. (1996) and the ciliate-specific primer set CS 322 F and EU929R of Puitika et al. (2007).

The *Histiobalantium natans viridis* sequences and reference sequences from the nucleotide sequence library (NCBI) were aligned with CLUSTAL X 1.83 (Thompson et al. 1997). Phylogenetic trees were generated using neighbour-joining (NJ), maximum parsimony (MP), and maximum likelihood (ML). NJ analysis was conducted using the program package MEGA 4

(Tamura et al. 2007). Distances were estimated by the NJ method with the TrN model of substitution (Tamura and Nei 1993) and with the assumption of rate heterogeneity among sites. The gamma-shaped parameters (alpha) were estimated with eight categories from PUZZLE version 5.2 (Strimmer and von Haeseler 1996). The proportion of invariable sites was 2.132 for the data sets. The transition/transversion ratio of the Hasegawa et al. (1985) model was estimated by maximizing the likelihood value for the NJ topology. The statistical significance of the tree branches was assessed by 1000 bootstrap resamplings (Felsenstein 1985). MP analysis was conducted using default settings in MEGA (Tamura et al. 2007). ML analysis was conducted using version 4.0b10 of PAUP* (Swofford 2002). The shape parameter of the gamma distribution was the same as used for the NJ analysis.

Accession numbers. The accession numbers of the SSU rDNA nucleotide sequences used for the phylogenetic analysis are given in the phylogenetic tree.

Results

Redescription of *Histiobalantium natans viridis* Kahl, 1931

1859 *Pleuronema natans* Claparède and Lachmann, Mem. Inst. natn. génev., 6 (year 1858): 276.
1886 *Histiobalantium agile* sp. nov. Stokes, Ann. Mag. nat. Hist. (Ser. 5), 17: 106 (supposed synonym).
1931 *Histiobalantium (Pleuronema) natans* (Clap. u. L., 1858) – Kahl, Tierw. Dtl., 21: 390 (review of genus).
1931 *Histiobalantium natans* forma *viridis* Kahl, Tierw. Dtl., 21: 390.
1972 *Histiobalantium natans* (Clap. & Lachm., 1858) – Dragesco and Iftode, Protistologica, 8: 347 (redescription from silvered specimens).
1989 *Gajewskiella macrostoma* Obolkina, Fauna Baikala, p. 5 (new synonym).

Size 70–100 × 35–55 μm in vivo, usually about 80 × 50 μm; not contractile. Specimens on average shrunken by at least 20% in protargol slides, as compared to measurements in vivo and silver nitrate preparations (osmium fixation!); length:width ratio also distinctly influenced by preparation procedures: 1.4–1.6 ($n = 10$) in vivo (view 2, see Table 1), while 1.7–2.4 in preparations, depending on method and side viewed (Table 1). Basically, slenderly to broadly ovate when seen ventrally/dorsally, while slenderly to broadly ellipsoidal when seen laterally or obliquely; slightly flattened ventrally and laterally, transverse view thus loaf-like (Fig. 3). Accordingly, three shapes occur (views 1–3, Table 1): narrowest when seen laterally (view 3, Figs 12, 30), widest when seen obliquely (view 2; ~45°; protargol preparations, Figs 1, 4, 11, 21–23, 26–28, 34, 35) or ventrally/dorsally (view 1, silver nitrate preparations; Figs 24, 25, 33, 36); view 2 most common because assumed when cells stand still and slightly project right mouth margin producing a small but quite typical convexity in or underneath mid-body (Figs 1, 11, 23–28, 34, 35); convexity indistinct or not recognizable in views 1 and 3.

Nuclear apparatus slightly above body centre (Figs 1, 5, 11, 12, 30, 34; Table 1). Macronucleus consisting of 1–8, on average 2 nodules; probably this is natural variability because specimens with one or many nodules are ordinary in all other main features, and post-conjugational reorganization is unlikely because conjugating cells did not occur. Individual nodules globular, invariably strongly wrinkled in silver preparations, while more or less smooth in vivo; probably usually connected by a short strand of nuclear material; fuse when cell is dividing; nucleoli not recognizable, either in vivo or in preparations. Most likely two micronuclei, one each in a small depression of macronucleus nodules, about 2–3 × 2 μm in size ($n = 10$), often difficult to recognize because not impregnated with the protargol method used and difficult to distinguish from similar-sized cytoplasmic inclusions. On average seven contractile vacuoles, four in left side, three in right. Excretory pores rather evenly spaced in a stripe between ciliary rows; rarely two pores/vacuole (Figs 1, 8, 32, 35, 36; Table 1). Cytopyge near posterior end of cell slightly right of midline of dorsal side, marked by an argyrophilic line and three shortened kineties, producing an oblong, unciliated area (Figs 5, 32). Extrusomes along left side of kineties, distinct in vivo because refractive and numerous, forming a rather distinct fringe (Figs 1, 21, 22, 29, 31); individual extrusomes slightly curved rods with rounded ends, 3.5–4.5 × 0.3 μm in size (Fig. 6); in silver nitrate preparations appear as distinct rings, about 1.5 μm wide, when docked and as minute granules when extruded (Fig. 7). Cortex rigid, specimens thus rather stiff.

Cytoplasm studded with greenish and brownish symbiotic algal chloroplasts of various shapes and 3–6 μm in size, some small lipid droplets, and many highly refractive crystals mainly in posterior body half (Figs 1, 21, 22). Freshly collected specimens greenish due to the algae contained; most algae without cell wall and thus likely cleptoplasts, as described by Esteban et al. (1997); most chloroplasts green, some brownish and thus likely originating from chrysophytes or dinoflagellates; cleptoplasts disappear after 1 week of laboratory cultivation without algae, making the up to 7 μm long crystals more distinct and posterior body half dark under transmitted light (Figs 24–28). Field specimens

Table 1. Morphometric data on *Histiobalantium natans viridis*.

Characteristics[a]	Method[b]	$\bar{x}$	M	SD	CV	Min	Max	*n*
Body, length	P	65.1	65.0	5.7	8.7	50.0	75.0	19
Body, length	CHL	82.8	81.0	8.3	10.0	67.0	95.0	17
Body, width (view 1)[c]	P	30.9	31.0	3.2	10.5	24.0	36.0	12
Body, width (view 1)[c]	CHL	49.7	50.0	6.0	12.1	38.0	62.0	11
Body, width (view 2)[c]	P	34.2	35.0	3.2	9.3	29.0	38.0	19
Body, width (view 2)[c]	CHL	46.2	46.0	3.5	7.6	40.0	52.0	13
Body, width (view 3)[c]	P	27.8	29.0	2.9	10.5	22.0	32.0	19
Body, width (view 3)[c]	CHL	39.8	39.5	3.6	9.0	35.0	46.0	12
Body length:width, ratio (view 1)[c]	P	2.1	2.1	0.2	10.8	1.9	2.7	12
Body length:width, ratio (view 1)[c]	CHL	1.7	1.7	0.2	10.0	1.5	2.1	13
Body length:width, ratio (view 2)[c]	P	1.9	1.9	0.2	10.5	1.6	2.5	19
Body length:width, ratio (view 2)[c]	CHL	1.8	1.8	0.2	13.2	1.3	2.1	11
Body length:width, ratio (view 3)[c]	P	2.4	2.4	0.3	13.9	1.7	3.0	19
Body length:width, ratio (view 3)[c]	CHL	2.2	2.1	0.5	20.2	1.6	3.1	12
Oral opening, length	P	32.8	33.0	3.5	10.5	26.0	42.0	19
Oral opening, length	CHL	39.9	38.0	6.9	17.2	30.0	63.0	17
Oral opening, width (view 1)[c]	P	9.4	8.5	2.7	28.4	5.0	15.0	18
Oral opening, width (view 1)[c]	CHL	9.4	9.5	3.4	36.5	5.0	15.0	8
Mouth, depth (view 3)[c]	CHL	17.4	17.0	2.6	15.1	15.0	22.0	8
Anterior body end to 1st AM, distance	P	17.2	17.0	2.4	14.0	13.0	22.0	19
Anterior body end to vertex of PM, distance	P	50.1	50.0	4.6	9.2	40.0	60.0	19
Anterior body end to MA figure, distance	P	15.7	15.0	2.6	16.7	12.0	21.0	19
Adoral membranelle 1, length	P	6.5	6.0	0.9	14.0	5.0	8.0	19
Adoral membranelle 2, length	P	10.1	10.0	1.3	12.6	8.0	12.0	19
Adoral membranelle 3, length	P	10.8	11.0	0.8	7.3	9.0	12.0	19
Macronucleus figure, length	P	25.8	26.0	3.7	14.4	20.0	32.0	19
Anterior macronucleus nodule, length	P	13.3	13.0	1.9	14.0	10.0	17.0	19
Anterior macronucleus nodule, width	P	9.6	10.0	1.9	6.0	15.0	19.6	19
Macronucleus nodules, number	P	2.4	2.0	1.2	50.8	1.0	8.0	29
Somatic kineties, number	P	57.2	57.0	3.6	6.3	52.0	65.0	19
Kinetids in a lateral kinety, number	P	53.4	52.0	5.9	11.1	40.0	65.0	19
Right side excretory pores, number	CHL	2.7	2.0	1.0	37.6	1.0	5.0	17
Left side excretory pores, number	CHL	4.1	4.0	0.8	19.0	2.0	5.0	17
Excretory pores, total number	CHL	6.8	7.0	1.6	23.0	3.0	10.0	17

[a]Data based on fixed, mounted, and silver-impregnated specimens from field; split material, that is, half each was used for protargol and silver nitrate impregnation. AM – adoral membranelle, CV – coefficient of variation in %, M – median, MA – macronucleus, Max – maximum, Min – minimum, *n* – number of specimens investigated, PM – paroral membrane, SD – standard deviation, $\bar{x}$ – arithmetic mean.

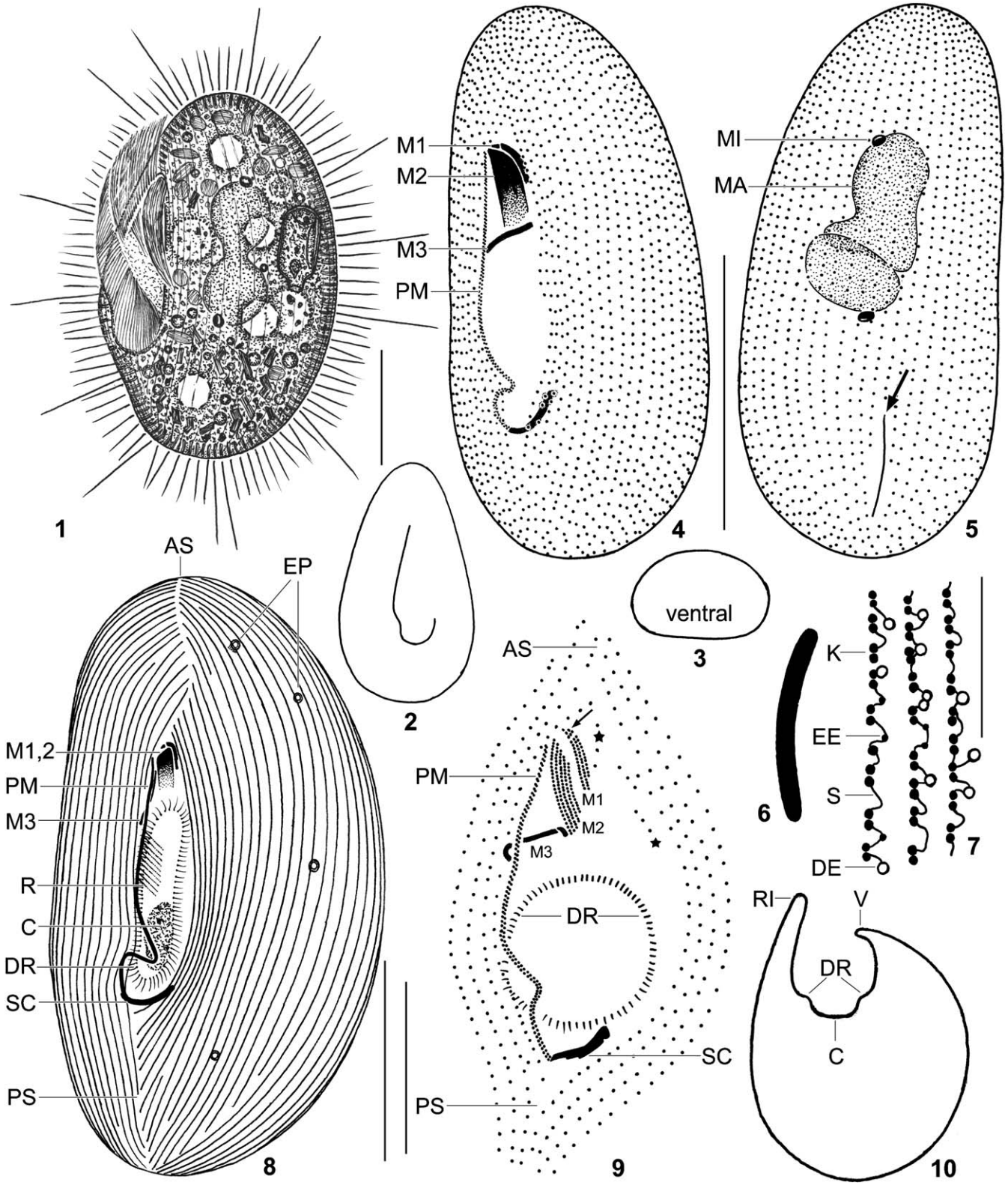
[b]CHL – Chatton–Lwoff silver nitrate impregnation as described in Foissner (1991), P – protargol impregnation of Bouin fixed specimens, protocol A in Foissner (1991).

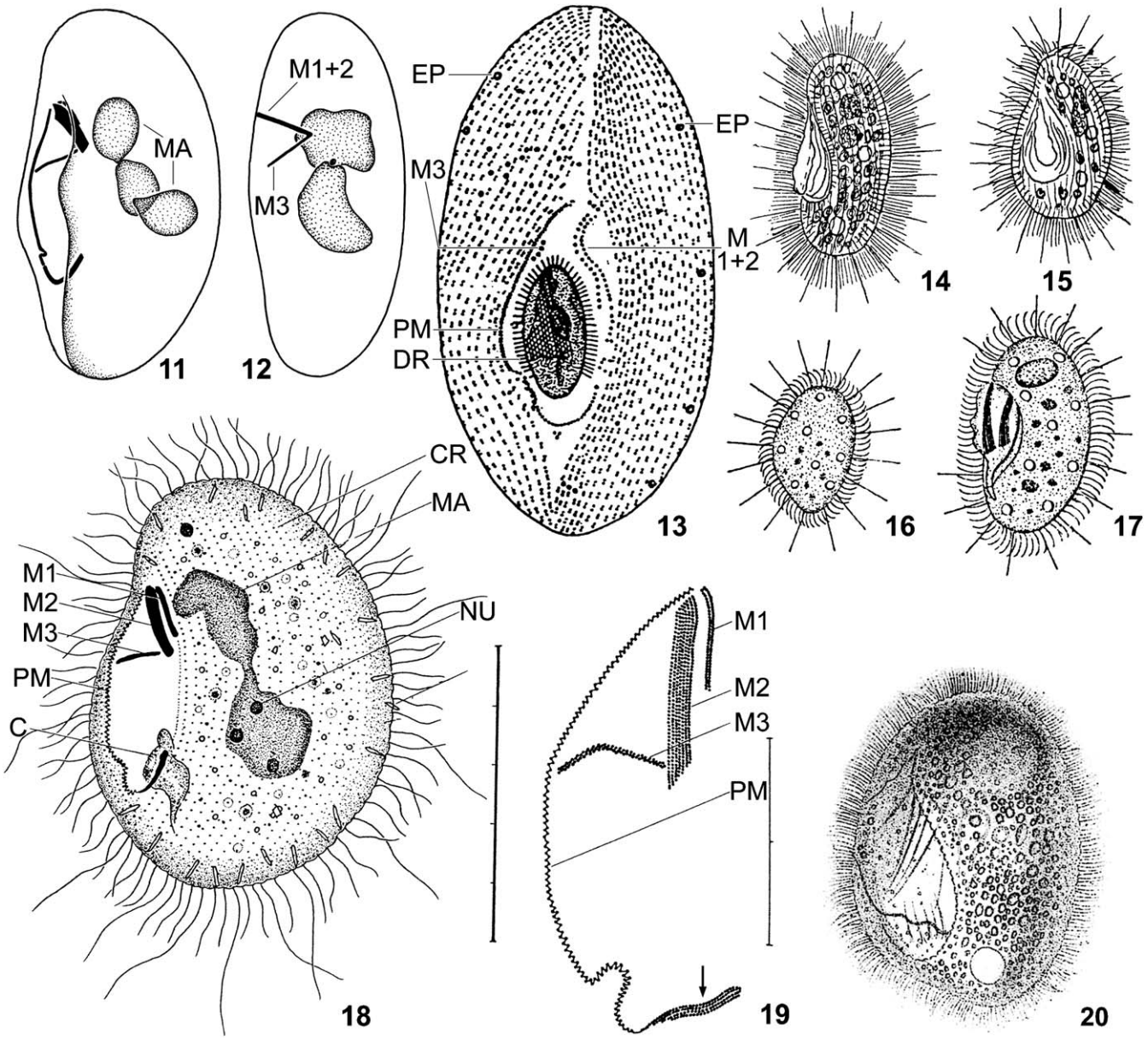
[c]View 1: see Figs. 33, 36; view 2: see Figs. 34, 35; view 3: see Fig. 30.

contain loricae of *Trachelomonas* (Euglenophyta) and some food vacuoles with granular contents, possibly from digested cleptoplasts (Fig. 1); do not grow well with bacterial food in the laboratory. Can swim rapidly to and fro, but soon stands still with spread cilia, collecting food with the long adoral cilia.

Figs 1–10. *Histiobalantium natans viridis* from life (1, 2, 3, 6), after protargol impregnation (4, 5, 9), and after silver nitrate preparation (7, 8, 10). **1.** Left side view of a representative specimen, length 80 μm. **2, 3.** Ventral and transverse outline. **4, 5.** Ventro- and dorsolateral view showing somatic and oral ciliary pattern. Arrow marks cytopyge. **6.** Extrusome, length 4 μm. **7.** Silverline pattern. Probably, one of the two granules composing a kinetid (K) is a parasomal sac. **8.** Ventral view of a specimen with narrow oral opening. Note shortened ciliary rows. **9.** Oral and circumoral ciliary pattern of a wide-mouthed specimen. Arrow marks anterior segment of adoral membranelle 1. Asterisks denote kinetofragments at left mouth margin. **10.** Transverse section at level of cytostome (scheme composed from several specimens). AS – anterior suture, C – cytostome, DE – docked extrusome, CR – ciliary rows DR – deep oral rod structure, EE – extruded extrusome, EP – excretory pores of contractile vacuoles, K – kinetid, MA – macronucleus, MI – micronucleus, M1, 2, 3 – adoral membranelles, PM – paroral membrane, PS – posterior suture, R – oral ribs, RI – right margin of oral opening, S – silverline, SC – scutica, V – velum (left mouth margin). Scale bars 30 μm (1, 4, 5, 8) and 10 μm (7, 9).

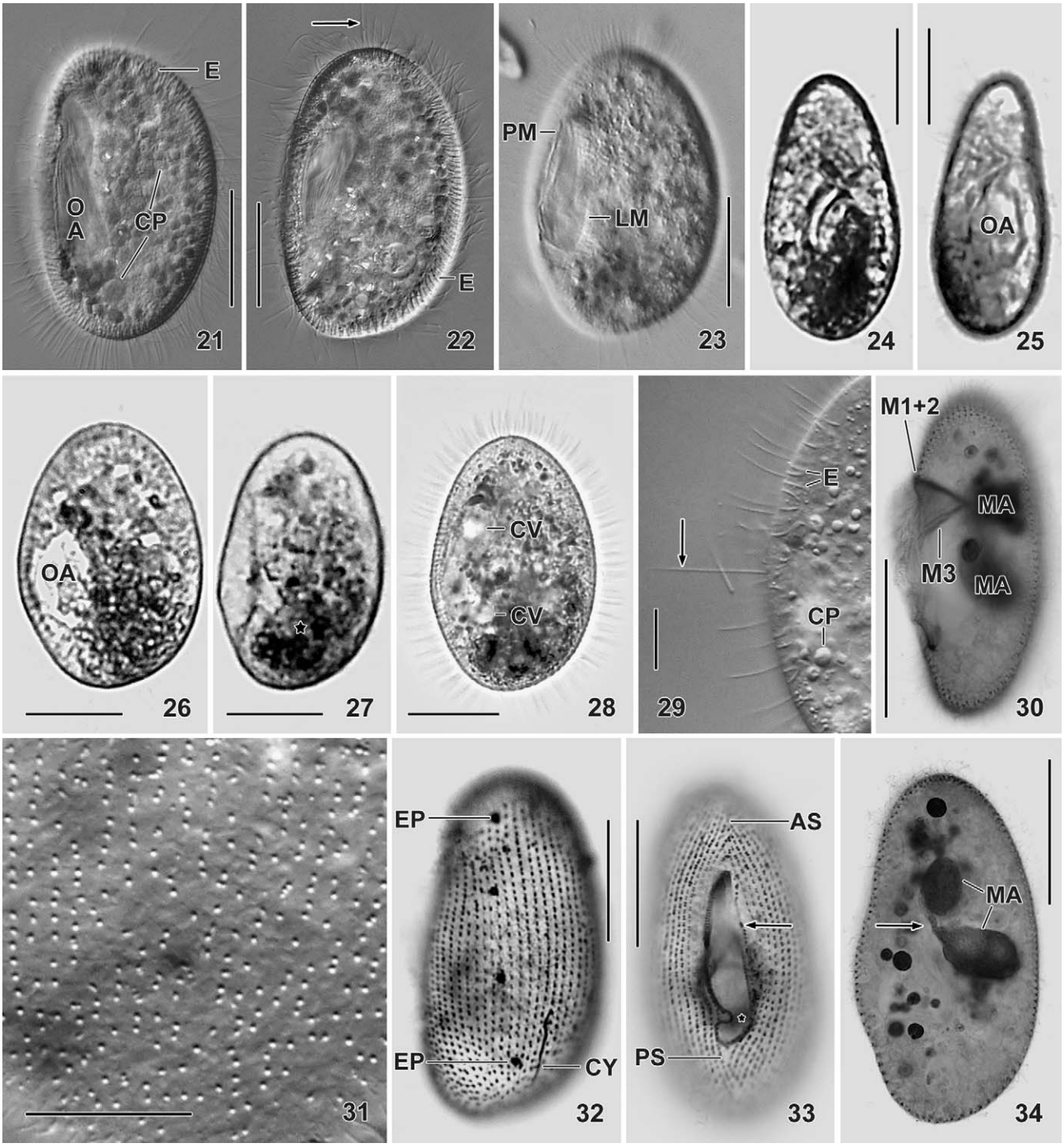
Somatic ciliature composed of monokinetids appearing as single, dark dots in protargol preparations (Figs 4, 5, 9), while paired in silver nitrate slides where possibly the parasomal sacs impregnate (Figs 35, 36). Many ordinary and some ''sensory'' cilia about 9 and 20 μm long, respectively; both spread in resting specimens (Figs 1, 21, 22, 29). Sensory cilia within ordinary ciliary rows, i.e., without specific features and thus not



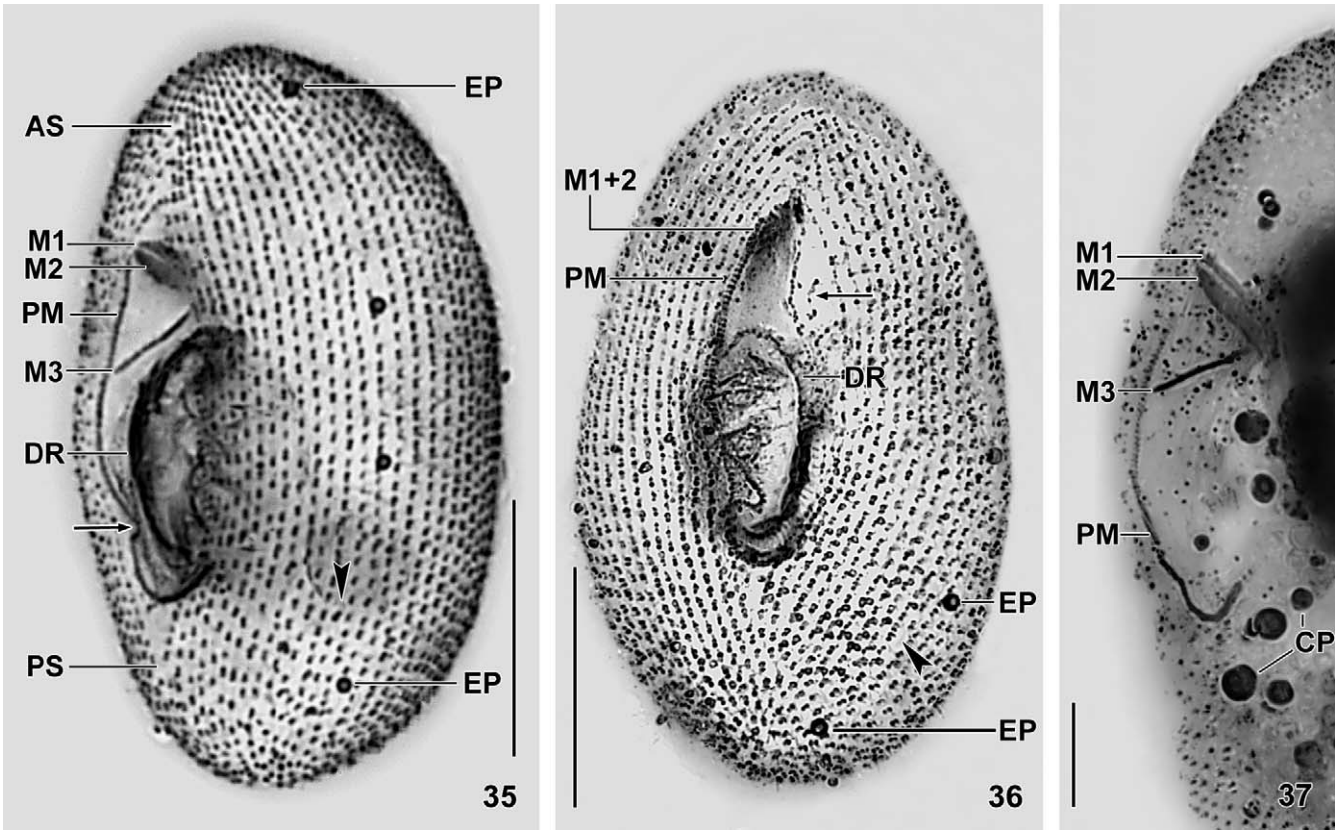


Figs 11–20. Various *Histiobalantium natans* populations. **11, 12.** *Histiobalantium natans viridis*, outline, oral apparatus, and macronucleus of protargol-impregnated Japanese specimens in view 2 and view 3 (see description), length 70 and 60 μm. **13.** *Gajewskiella macrostoma*, ventral view of a silver nitrate-impregnated specimen, length 80 μm (from Obolkina 1989). Labels are as in the original, except the deep oral rod structure (DR) which was added by the authors. This ciliate is a junior synonym of *Histiobalantium natans* (cp. Fig. 36!). **14, 15.** *Histiobalantium natans nigricans* (80 μm) and *H. natans viridis* (70 μm) in vivo (from Kahl 1931). **16, 17.** *Histiobalantium agile*, length about 70 μm, is probably a junior synonym of *H. natans* (from Stokes 1886). **18, 19.** *Histiobalantium natans*, composite from live and silver preparations (18) and oral apparatus after protargol impregnation (19); scale bar divisions 10 μm (from Dragesco and Iftode 1972). Arrow marks scutica. **20.** *Pleuronema natans*, size not given (from Claparède and Lachmann 1859). C – cytostome, CR – ciliary rows, DR – deep oral rod structure, EP – excretory pores of contractile vacuoles, MA – macronucleus, M1, 2, 3 – adoral membranelles, NU – nucleolus, PM – paroral membrane.

recognizable in silver preparations. Cilia arranged in an average of 57 narrowly spaced and densely ciliated, meridional rows, forming a preoral and postoral suture; both sutures indistinct because right and left side kineties abut without leaving a blank stripe (Figs 4, 8, 9, 33, 35; Table 1). Ciliary rows more densely spaced around oral opening, usually an ordinary kinety along flexible left mouth margin (Figs 4, 8, 35), rather frequently some scattered granules, possibly remnants from extruded trichocysts or small kinety fragments forming short rows (Figs 9, 33, 36). Many ciliary rows slightly shortened posteriorly, some anteriorly and along paroral membrane; short kinetofragments may be interspersed between ciliary rows, especially in broader



Figs 21–34. *Histiobalantium natans viridis*, Japanese specimens from life (21–29, 31), after protargol preparation (30, 34), and after silver nitrate impregnation (32, 33). **21–23, 26–28.** Left side views (''view 2'') of resting specimens in interference contrast (21–23) and bright field (26–28), showing shape variability, the location of the oral apparatus, the tactile cilia (Fig. 22, arrow), and the posterior crystal accumulation (Fig. 27, asterisk). **24, 25.** Ventral views (''view 1''). **29.** *Histiobalantium* has about 9 μm long ordinary cilia and up to 20 μm long tactile cilia (arrow). **30.** Narrow-side view (''view 3'') showing the ''V'' formed by the adoral membranelles. **31.** Surface view showing the extrusome rows. **32.** Dorsolateral view (''oblique view 2'') showing the cytopyge and four excretory pores. **33:** Ventral view (''view 1'') showing a ''disturbed'' ciliary row on left margin of oral opening (arrow) and the characteristic notch (asterisk) of the paroral membrane. **34.** Outline (''view 2'') showing the characteristic convexity in oral region and the two macronucleus nodules connected by a thick strand of argyrophilic material (arrow). AS – anterior suture, CP – cleptoplasts, CV – contractile vacuoles, CY – cytopyge, E – extrusomes, EP – excretory pores, LM – left margin of oral opening, MA – macronucleus nodules, M1, 2, 3 – adoral membranelles, OA – oral apparatus, PM – paroral membrane, PS – posterior suture. Scale bars 10 μm (Figs 29, 31) and 30 μm (21–28, 30, 32–34).



Figs 35–37. *Histiobalantium natans viridis*, Japanese specimens after silver nitrate (35, 36) and protargol (37) impregnation. **35.** Left side view (“view 2”) of a representative specimen, showing important organelles, such as the oral apparatus and the arrangement of the somatic ciliary rows. The arrow marks the characteristic notch in the paroral membrane, while the arrowhead denotes a posterior kinetofragment. **36.** Ventral view of a *Gajewskiella*-like specimen (cp. Fig. 13) with wrinkled left mouth margin (arrow), a preparation artifact feigning adoral membranelles (Fig. 13) which, however, are attached to the anterior mouth margin. The arrowhead marks a shortened ciliary row. **37.** Oral structures and cleptoplasts. AS – anterior suture, CP – cleptoplasts, DR – deep oral rod structure, EP – excretory pores of the contractile vacuoles, M1, 2, 3 – adoral membranelles, PM – paroral membrane, PS – posterior suture. Scale bars 30 μm.

posterior body half (Figs 4, 5, 8, 35, 36). Silverline pattern as in other scuticociliates, i.e., a rather wrinkled silverline connects basal bodies within rows and with the extrusomes, which appear as minute rings and granules when docked or just released, respectively (Fig. 7).

Oral apparatus in central quarters of cell, i.e., about half body length (Table 1). Oral opening fusiform, width varies strongly, i.e., from 5 to 15 μm both in protargol and silver nitrate preparations, indicating that it can be closed (Figs 1, 4, 8, 9, 25, 26, 33, 35, 36). Buccal cavity perfectly boat-shaped, on average 17 μm deep at cytostomial opening; cavity bottom occupied by two prominent organelles more distinct in silver nitrate than protargol preparations: (i) the “deep oral rod structure”, a new term for a large, elliptical area bounded by about 5 μm long, narrowly spaced rods (fibres?) called “stries orales” by the French scientists and (ii) the elliptical, rather large cytostome in posterior half of the deep oral rod structure (Figs 8–10, 35, 36). Adoral membranelles in anterior third of buccal cavity, cilia 20–25 μm long, membranelles 1 and 2 attached to oblique anterior portion of cavity (boat), membranelle 3 on right wall of cavity, forming a highly characteristic V-shaped pattern with membranelles 1 and 2 (Figs 1, 4, 8, 9, 12, 30, 35, 36, 37). Membranelle 1 about 6 μm long, composed of a minute, triangular anterior portion separated by a minute cleft from the longer posterior portion, three-rowed with left row slightly shortened posteriorly. Details of membranelle 2 difficult to recognize, about 10 μm long, probably composed of 5 or 6 ciliary rows slightly decreasing in length from right to left. Membranelle 3 on average 11 μm long, composed of two deeply impregnating lines, probably ciliary rows, separated by a lighter stained centre (Figs 9, 35, 37). Paroral membrane occupies convex right margin of oral opening, possesses a unique shape, i.e., a long, straight anterior portion is followed by a deep notch and a semicircular segment surrounding the posterior mouth margin (Figs 1, 4, 8, 9, 35–37); composed of zigzagging dikinetids with about 13 μm long cilia, except for homogenously impregnating posterior portion (scutica) probably consisting of three or more ciliary rows, as

indicated by the sometimes staggered margin of the membrane base and its widening from 0.5–1 μm anteriorly to 1–1.5 μm posteriorly; associated with faintly impregnated oral ribs recognizable only in silver nitrate slides (Fig. 8).

Molecular analysis

A preliminary analysis of the SSU rDNA nucleotide sequences showed a close relationship of *Histiobalantium* with the class Oligohymenophorea and the subclass Scuticociliatia, as defined by Lynn and Small (2002). Of the rather many scuticociliatid sequences available in public databases, we selected those which are likely to be based on correctly identified material, at least to genus level. As an outgroup, we choose three other oligohymenophorean subclasses, viz., the Hymenostomatia (*Tetrahymena*, *Colpidium*), the Peniculia (*Paramecium*), and the Peritrichia (*Opisthonecta*).

The Scuticociliatia form a monophyletic assemblage with 97–99% bootstrap support (Fig. 38). Within the Scuticociliatia four groups are recognizable, of which one is formed by the families Cyclididiidae and Pleuronematidae to which *Histiobalantium* belongs. The pleuronematid cluster, which has 100% bootstrap support, contains the genera *Histiobalantium*, *Schizocalyptra*, and *Pleuronema*. *Cyclidium plouneouri* and *C. glaucoma* are related to the Pleuronematidae with 93–96% and 89–91% bootstrap support, respectively (Fig. 38).

Discussion

Synonymy, identification, nomenclature

According to Kahl (1931), *Pleuronema natans* Claparède and Lachmann, 1859 has a junior synonym: *H. agile* Stokes, 1886. We agree at the present state of knowledge, although the description of Claparède and Lachmann (1858) is meagre and Fig. 20 likely shows a rather distorted specimen. Indeed, the identity of the European and North American populations is questionable because Stokes (1886) describes and illustrates many scattered contractile vacuoles and does not give any indication of coloured inclusions (cleptoplasts), so typical for European and Asian field populations of *H. natans* (Figs. 16 and 17). Thus, a detailed investigation of a North American population is required to substantiate or disprove the synonymy suggested.

Kahl (1931) split *H. natans* in a forma *viridis* (with algal food, body broad and flat when motionless, stands still under coverslip, subsapropelic) and a forma *nigricans* (with dark = refractive food (?) inclusions, body slender, does not rest under coverslip, strongly sapropelic). The population from Biwa Lake matches

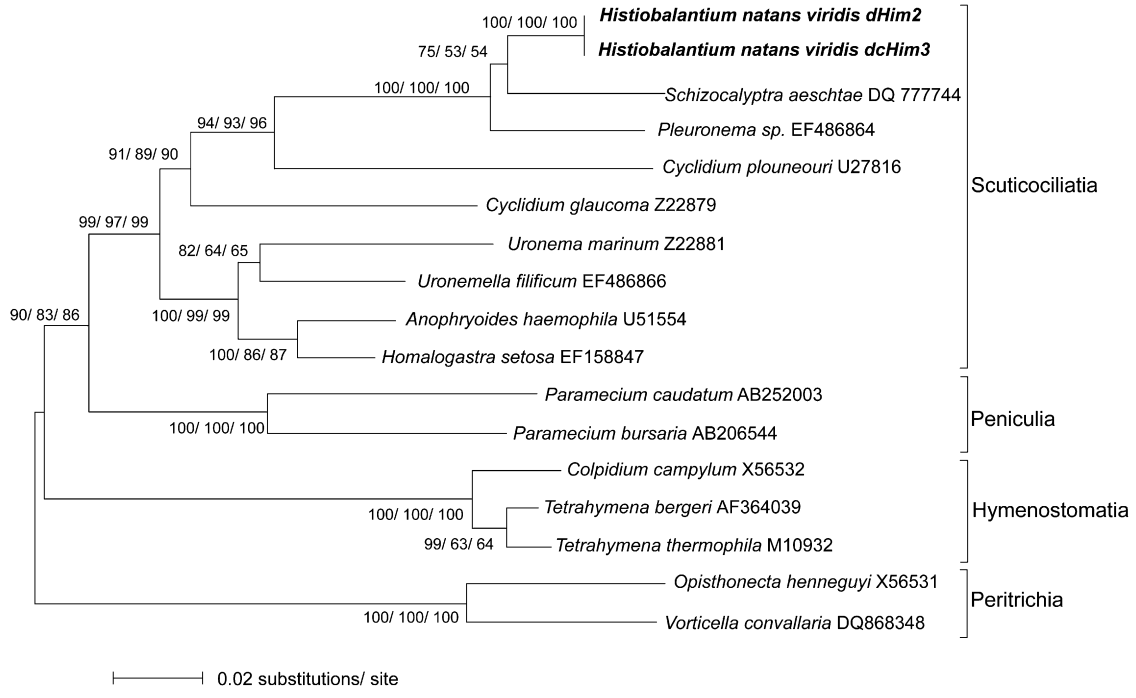


Fig. 38. Neighbour-joining phylogenetic tree based on the small subunit (SSU) rDNA sequence of *Histiobalantium natans virdis* and other ciliates. The scale bar indicates the genetic distance. Numbers at branching points show bootstrap values of 1000 replicates each for three methods: neighbour-joining (NJ), maximum parsimony (MP), and maximum likelihood (ML). GenBank numbers follow species names.

the forma *viridis* (cp. Figs 15, 21–23, 26–28). However, after a week of laboratory cultivation, the specimens have digested the cleptoplasts and become colourless, while other features do not change (field specimens, Figs 21–23; laboratory specimens, Figs 26–28). Dragesco and Iftode (1972) used cultivated specimens and thus did not deal with this problem. One cannot exclude that the strongly sapropelic forma *nigricans* represents a distinct (sub)species as long as an accurate redescription is lacking. However, the dark appearance could be caused by masses of refractive crystals, some of which are present also in the forma *viridis* (Figs 1, 27).

Esteban et al. (1997) treated Kahl's forma *viridis* as a distinct species: "*Histiobalantium viridis*: an aerobic scuticociliate…". However, no evidence for this rank elevation is provided, except for the cleptoplasts. According to article 45.6.4 of the International Commission on Zoological Nomenclature (1999), Kahl's forma can obtain subspecies or infrasubspecies rank because Kahl (1931) did not fix the rank. We prefer subspecific rank at the present state of knowledge.

Comparison of the European and Asian *H. natans*

The foregoing discussion might appear too sophisticated. However, the problem is complex because our observations hardly match the redescription of *H. natans* by Dragesco and Iftode (1972). There are main differences in the structure of adoral membranelles 1 and 2 (M1, 2). According to Dragesco and Iftode (1972), M1 consists of two rows of basal bodies (Fig. 19), while the Lake Biwa population has three rows, of which the left row is shortened (Fig. 9). Further, M1 of the Asian population has a triangular anterior segment (Figs 9, 35), possibly lacking in the French population (Fig. 19; but see below). Membranelle 2 is obliquely truncate to the right in the French specimens (Fig. 19), while to the left in the Asian ones (Fig. 9). Further M2 is probably distinctly shorter in the Asian (10 μm; Table 1) than the French population (20 μm in Fig. 19, but only 11–13 μm in four other drawings, indicating error in Fig. 19). Dragesco and Iftode (1972) do not mention the deep oral rod structure; possibly, it did not impregnate.

Usually, such differences in the oral structures are considered as sufficient to separate populations at species level, especially because M1 of *H. bodamicum* Krainer and Müller, 1995 is structured as described by Dragesco and Iftode (1972) for *H. natans*. On the other hand, *H. majus* and *H. minor* have M1 structured as our population of *H. natans* (Grolière 1973; Wilbert 1986). Thus, there is no easy way to decide the status of the *Histiobalantium* from Lake Biwa. Is it a distinct species or is the redescription of the European *H. natans* deficient? We tend to assume the latter because Dragesco and Iftode (1972) illustrate, but do not comment on, the anterior segment of M1 in early and mid-dividers. Further, sizes are probably incorrect (see above). However, we cannot exclude that Dragesco and Iftode (1972) investigated the forma *nigricans*, which then would be a distinct species. Obviously, the European *H. natans* is in urgent need of detailed reinvestigation.

Systematic position and family affiliation of *Histiobalantium*

The sequence data show that *Histiobalantium* belongs to the Scuticociliatia, where it forms a strongly supported clade with *Schizocalyptra* and *Pleuronema* (Fig. 38). This is endorsed by distinct morphological similarities between these genera (Dragesco 1968; Wilbert 1986; present study), especially the occurrence of a deep oral rod structure in *Histiobalantium* and *Schizocalyptra*. However, this classification contrasts with that of Dragesco and Iftode (1972), who studied the ontogenesis of *H. natans* and concluded that *Histiobalantium* is more closely related to the peniculine than pleuronematine hymenostomes. Recently, Ma et al. (2005) supported this conclusion by a phylogenetic analysis, presumably using the data of Dragesco and Iftode (1972).

Dragesco and Iftode (1972) could not find a scutica in *H. natans*, while Grolière (1973) observed a single divider of *H. majus*, suggesting as scutica the thickened proximal end of the paroral membrane. Later, this has been corroborated by comparative ontogenetic studies in *Pleuronema* (Grolière and Detcheva 1974; Ma et al. 2003). Obviously, Dragesco and Iftode (1972) missed some early ontogenetic stages in their study. Thus, we recognize *Histiobalantium* as a "good" scuticociliate closely related to *Schizocalyptra* and *Pleuronema*.

According to Puytorac and Corliss (Corliss 1979; de Puytorac 1994), *Histiobalantium* represents a monotypic family (Histiobalantiidae) within the order Pleuronematida, while *Pleuronema*, *Schizocalyptra* and *Pleurocoptes* constitute the family Pleuronematidae. This classification has been accepted by Lynn and Small (2002), but is hardly supported by the ontogenetic (see above) and molecular (Fig. 38) data, which show a close relationship of *Histiobalantium* and *Schizocalyptra*/*Pleuronema*. However, there is a curious feature which indicates that *Histiobalantium* might indeed deserve a distinct family: the location of the cytopyge in the dorsolateral area of the cell (Figs 5, 32; Wilbert 1986). To our best knowledge, this is unique among the scuticociliates and difficult to understand because there is sufficient space for a cytopyge postorally, where it is found in most or even all other oligohymenophoreans.

The Cyclidiidiidae seem to be the nearest relatives of the Pleuronematidae/Histiobalantiidae. However, they

are paraphyletic according to the SSU rDNA nucleotide sequences (Fig. 38). This is not surprising, considering their conspicuous morphological diversity (Lynn and Small 2002; Foissner et al. 2002), possibly resulting from convergent evolution of different ancestors.

Based on data from Obolkina (1995), Jankowski (2007) classified *Histiobalantium* Stokes, 1886, *Sulcigera* Gajevskaja, 1928, and *Gajewskiella* Obolkina, 1989 in the family Sulcigeridae Gajewskaja, 1933, while *Pleuronema* Dujardin, 1841, *Pleurocoptes* Wallengren, 1896, *Schizocalyptra* Dragesco, 1968, and *Hippocomos* Czapik and Jordan, 1977 were united in the family Pleuronematidae Kent, 1881. While the classification of *Hippocomos* is possibly correct because it seems to have a deep oral rod structure (Czapik and Jordan 1977; present paper), *Gajewskiella* Obolkina, 1989 is very likely a junior synonym of *Histiobalantium* (see next chapter), and the redescription of *Sulcigera comosa* Gajevskaja, 1928 is possibly based on a misidentified *Histiobalantium* related or even identical with *H. bodamicum* Krainer and Müller, 1995. Such interpretation is justified because Obolkina's ciliate does not show the two most conspicuous organelles of *S. comosa*, viz., four cirrus-like posterior appendages up to 70 µm (!) long and two semicircular arrays of very long cilia on the anterior pole area (Gajewskaja 1933).

The genus *Gajewskiella* Obolkina, 1989

Obolkina (1989) found a supposedly undescribed ciliate in the littoral mud of Lake Baikal (Fig. 13). However, the new ciliate, *Gajewskiella macrostoma*, which has been observed only in silver nitrate preparations, is very likely a junior synonym of *H. natans*. This is indicated by distinct similarities in size and shape (80 × 45 µm, ellipsoidal), the number and arrangement of the contractile vacuoles (12–14 lateral excretory pores), the number of ciliary rows (50–55), and the large mouth with a deep oral rod structure (see description of *H. natans*). Obolkina (1989) obviously overlooked adoral membranelles 1 and 2, which are attached to the anterior end of the oral opening (Figs 4, 8, 33, 36). She designated as membranelles 1 and 2 some minute granule rows along the right mouth margin. However, these are small somatic fragments or extrusome rows found also in some *H. natans* specimens (Fig. 36). Membranelle 3 of *G. macrostoma* is located as in *H. natans*, but only the distal region has been recognized (Fig. 13). Synonymy becomes obvious when Figs 13 and 36, which are based on the same preparation method, are compared.

Acknowledgments

Financial support was provided to W. Foissner by Prof. Dr. Tadao Takahashi, Nishikyusyu University, on behalf of the Japanese Society of Protozoologists; the Lake Biwa Museum, Comprehensive Research Project 06–02; the MEXT, Kakenhi (Grant 18770060 to S. Shimano); and the Austrian Science Foundation (Grant P19699–B17 to W. Foissner). We greatly acknowledge the technical assistance of Mag. Barbara Harl, Mag. Gudrun Fuss, Robert Schörghofer, and Andreas Zankl. Special thanks to Dr. Mark Grygier, organizer of the Lake Biwa biodiversity studies, for having a critical look at the manuscript.

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