Heterotrophic flagellates and centrohelid heliozoans from marine waters of Curacao, the Netherlands Antilles

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Abstract

Recent progress in understanding the early evolution of eukaryotes was tied to morphological identification of flagellates and heliozoans from natural samples, isolation of their culture and genomic and ultrastructural investigations. These protists are the smallest and least studied microbial eukaryotes but play an important role in the functioning of microbial food webs. Using light and electron microscopy, we have studied the diversity of heterotrophic flagellates and centrohelid heliozoans from marine waters of Curacao (The Netherlands Antilles), and provide micrographs and morphological descriptions of observed species. Among 86 flagellates and 3 centrohelids encountered in this survey, five heterotrophic flagellates and one centrohelid heliozoan were not identified even to the genus. Some flagellate protists have a unique morphology, and may represent undescribed lineages of eukaryotes of high taxonomic rank. The vast majority (89%) of identified flagellates is characterized by wide geographical distribution and have been reported previously from all hemispheres and various climatic regions. More than half of the species were previously observed not only from marine, but also from freshwater habitats. The parameters of the species accumulation curve indicate that our species list obtained for the Curacao study sites is far from complete, and each new sample should yield new species.

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Introduction

Heterotrophic flagellates and centrohelid heliozoans are the smallest and least studied groups of protists at both the morphological and molecular levels. Heterotrophic flagellates – the collective name for an extremely diverse "hodgepodge" of polyphyletic, colorless protists moving or feeding with flagella at least in one stage of their life cycle (Patterson and Larsen 1991) – are characterized by a significant variety of metabolism and ecology. At the same time, centrohelid heliozoans (Centroplasthelida Febvre-Chevalier and Febvre, 1984) is a monophyletic group of predatory protists, related to haptophyte algae within the supergroup Haptista Cavalier-Smith, 2003 (Burki et al. 2016). Centrohelids are characterized by the presence of surface cytoskeletal structures – siliceous scales or organic spicules, having an important diagnostic value. Heterotrophic flagellates and heliozoans are widespread in different types of freshwater and

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Abbreviations: SEM, scanning electron microscopy; TEM, transmission electron microscopy; LM, light microscopy; PhC, phase contrast; DIC, differential interference contrast.

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marine biotopes and play an important role in the functioning of the microbial food webs, thus providing effective pathways for the transformation of matter and energy in aquatic ecosystems (Arndt et al. 2000; Domaizon et al. 2003; Kiss et al. 2009).

Faunistic investigations of heterotrophic flagellates and heliozoans supported with morphological descriptions were especially common in protistological literature in the 90 s – early 2000s (Larsen and Patterson 1990; Lee and Patterson 2000; Lee et al. 2003; Mikrjukov 2001; Patterson and Simpson 1996; Patterson et al. 1993; Tong 1997b, c; Vørs 1992). At present, such studies are relatively rare and have been largely replaced by molecular surveys from bulk microbial community DNA (Geisen et al., 2019; Pawlowski et al., 2016). However, several recent advances in the study of eukaryotic evolution have been specifically tied to morphological identification of flagellates and heliozoans in natural samples, their isolation into culture, and genomic and ultrastructural investigations of such cultures. Morphological recognition and identification (sometimes approximate) of these protists in samples is precisely based on such "classical" studies, and would not have been possible without these. For example, recent recognition, isolation, and investigation of previously understudied or unknown heterotrophic flagellates was essential in addressing major evolutionary problems, such as the origins of photosynthesis and parasitism and the trajectory of plastid spread (Gawryluk et al. 2019; Janouškovec et al. 2015; Tikhonenkov 2020b), the origin of multicellular animals (Hehenberger et al. 2017; Tikhonenkov et al. 2020a), the evolution of mitochondrial genomes, and the rooting of the tree of eukaryotes and clarification of their relationships (Janouškovec et al. 2017; Lax et al. 2018; Strassert et al. 2019). Identification and establishing of clonal cultures of centrohelids was crucial for transcriptomic research and untangling the early diversification of eukaryotes (Burki et al. 2016).

Descriptions of many flagellates and heliozoans were made in the late XIX and early XX centuries using imperfect light microscopy. Many of these species lack type material, ultrastructural, and molecular data, and the taxonomy of many species and groups is in need of revision (Lee et al. 2003; Schoenle et al. 2020). Currently, the described diversity of these protists represents only a small fraction of their total species richness in nature (Cavalier-Smith and von der Heyden 2007; Corliss 2002). Metagenomic and metabarcoding sequencing of environmental samples has revealed several lineages representing high levels of hidden diversity: e.g., ribogroups MALV, MAST, MAOP, MAFO, deep-sea pelagic diplonemids (DSPD), or eupelagonemids (del Campo and Ruiz-Trillo, 2013; del Campo et al., 2015; Guillou et al. 2008; Massana and Pedrós-Alió 2008; Okamoto et al. 2019; de Vargas et al., 2015), as well as a great variety of small new phylogenetic lineages associated with almost all large eukaryotic groups (del Campo et al., 2016; Keeling and Campo, 2017). New species of flagellates and centrohelids

are being discovered constantly, which is indicative of a poor state of exploration and insufficient sampling.

At the same time, the issues of the geographical distribution of protists are subject to lively, but stubbornly unresolved debate (Azovsky et al. 2016). Are protists species widespread around the globe, or is their distribution, like those of macroorganisms, more governed by the rules of historical biogeography? There are two alternative points of view on this issue: the Ubiquity Model (Fenchel and Finlay 2004; Finlay et al. 1996) and Moderate Endemicity Model (Foissner 2004, 2008). Additional data are needed to resolve this issue, especially in those regions on the planet that remain unexplored.

Here we describe a microscopical investigation of the species diversity of heterotrophic flagellates and centrohelid heliozoans in marine waters of the coast of Curacao, which has not specifically been investigated previously. Only one species (*Choanoeca flexa* Brunet et al., 2019) was described from the coastal waters of Curacao (Brunet et al. 2019). Thirty six species of heterotrophic flagellates and one centrohelid heliozoan (*Heterophrys myriopoda* Archer, 1869) are known from the Caribbean Sea in general (Thomsen and Østergaard 2019; Thomsen et al. 2020; Vørs, 1993b) until recently. We find the diversity of heterotrophic protists on Curacao to be rich and intriguing, including the presence of species potentially important for clarifying previously puzzling evolutionary and ecological questions. We describe a survey of light and electron microscopical studies of the diversity of heterotrophic flagellates and centrohelid heliozoans from marine waters of several locations on Curacao, and provide micrographs and morphological descriptions of observed species, as well as discussing their distribution and potential importance.

Material and methods

Marine water samples were taken from eight locations around the island of Curacao in April 2018. Samples came from several biotopes (from the surface of corals and sponges, *Sargassum* algae wrings, sand, coral sand, and water column) both nearshore and at depths of 12–25 m (detailed descriptions of sampling points are given in Table 1). From each biotope, several replicates were taken, which were subsequently summarized. A total of 52 samples were investigated microscopically. Water temperature in the studied region is relatively constant, about 26–29 °C. Samples were placed into 50 mL plastic tubes and transported to the laboratory at 4 °C. Samples were enriched with a suspension of *Pseudomonas fluorescens* Migula, 1895 bacteria, and placed in Petri dishes. Samples were kept at 22 °C in the dark and observed for 10 days to reveal the cryptic species diversity (Tikhonenkov et al., 2008a; Vørs 1992). A culture of the kinetoplastid flagellate, *Procryptobia sorokini* (Zhukov, 1975) Frolov et al., 2001, was used as a food source for predatory heliozoans and flagellates.

Table 1. Characteristics of sampling points.

Habitat	Coordinates, N, W	Date	Biotope	Salinity, ‰	Depth, m
1. Turtle lagoon Boka Ascension	12°16'22.0" 69°03'14.0"	April 20, 2018	1a. Interstitial sand water 1b. Sand	35	0
2. The eastern point of the Curacao island	12°12'32.3" 68°48'58.8"	April 24, 2018	2a. Sponge 2b. Coral 2c. Coral sand 2d. Sand 2e. *Sargassum* algae wrings	35	24.7
3. Bay Santa Martha	12°16'05.1" 69°07'43.7"	April 26, 2018	3a. Sponge 3b. Coral 3c. Sand	35	20
			3d. Coral sand	35	0
4. "Water Factory" sampling site near water treatment plant	12°06'34.0" 68°57'14.9"	April 19, 2018	4a. Sponge 4b. Coral 4c. Sand 4d. Water column	35	12–18
5. The western point of the Curacao island	12°22'31.7" 69°09'29.7"	April 26, 2018	5a. Sponge 5b. Coral 5c. Sand	35	20
6. Reef near the CARMABI research station	12°07'19.8" 68°58'09.4"	April 22, 2018	6. Coral	35	20
7. Hilton Hotel Beach	12°07'17.3" 68°58'08.8"	April 24, 2018	7. Coral sand	35	0
8. Hypersaline lagoon Saliña Sint Maria	12°12'45.7" 69°03'15.3"	April 20, 2018	8a. Black clay 8b. Water column	52	0

An AxioScope A1 upright light microscope (Carl Zeiss, Germany) with DIC and phase contrast and water immersion objectives (total magnification ×1120) was used for observations of living cells. Electron microscope preparations were carried out according to described methods (Mikrjukov 2002; Moestrup and Thomsen 1980) and observed in a JEM–1011 (Jeol, Japan) transmission electron microscope and a JSM–6510 LV (Jeol, Japan) scanning electron microscope.

The dendrogram showing the similarity of types of biotopes by flagellate species composition was drawn on the basis of the Dice similarity index using the paired group algorithm in the PAST software package (Hammer et al. 2001).

The analysis of the geographic distribution of heterotrophic flagellates was based on the "morphospecies" concept (Fenchel and Finlay 2006; Finlay et al. 1996) and carried out using the previously published database (Azovsky et al., 2016, 2020) and available literature sources. Only morphology-based (or morphology-confirmed) data were considered.

Results

Eighty-six species and forms of heterotrophic flagellates and three species of centrohelid heliozoans were observed (Table 2) and listed systematically according to the system of eukaryotes by Adl et al. (2019). This system operates hierarchical nameless ranks that ignore endings of clade names. Thus, genera grouped into a clade represent a family (Adl et al., 2019). A system of asterisks was used to identify levels of taxonomic ranks from high to low (the more asterisks, the lower the rank of the taxon). Morphological characteristics of most unusual and rare species of flagellates and all centrohelids are listed below.

AMORPHEA Adl et al., 2012
Obazoa Brown et al., 2013
*Apusomonadida Karpov and Mylnikov, 1989
Thecamonas sp. (Fig. 1a–c)

Observations: Cells are oval or reniform in shape, dorsoventrally flattened, 5–6 μm in length, 3.5–4.0 μm in width. The anterior flagellum is about the cell length, its basal part passes in the sleeve, the distal part is acronematic. The posterior flagellum is 4 times longer than the cell, along half-length passes in the sleeve. Numerous granules are visible inside the cell. The cells move by gliding, with anterior flagellum beating with a flicking motion. Observation based on description of six cells in LM.

Remarks: Cavalier-Smith and Chao (2010) divided marine *Amastigomonas*-like species into four genera: *Manchomonas*, *Multimonas*, *Podomonas*, and *Thecamonas*. Larsen and Patterson (1990) earlier introduced the last genus

Table 2. Species diversity of heterotrophic flagellates and centrohelid heliozoans of Curacao.

AMORPHEA Adl et al., 2012
Obazoa Brown et al., 2013
*Apusomonadida Karpov and Mylnikov, 1989
Amastigomonas debruynei De Saedeleer, 1931[c] – 2e, 3a, 3c, 8a
Podomonas griebenis (Mylnikov, 1999) Cavalier-Smith in Cavalier-Smith and Chao, 2010[c] – 3a, 5b, 8b
Thecamonas mutabilis (Griessmann, 1913) Larsen and Patterson, 1990[c] – 2a, 6
Thecamonas sp. – 7
*Opisthokonta Cavalier-Smith, 1987
**Holozoa Lang et al., 2002
***Filasterea Shalchian-Tabrizi et al., 2008
Ministeria vibrans Tong, 1997[c] – 6, 8b
***Choanoflagellata Kent, 1880–1882
****Craspedida Cavalier-Smith, 1997
*****Salpingoecidae Kent, 1880–1882 *sensu* Nitsche et al., 2011
Salpingoeca infusionum Kent, 1880–1882[c] – 1b, 4d
S. minor Dangeard, 1910[c] – 5a
S. pyxidium Kent, 1880–1882[c] – 4d
S. ringens Kent, 1880–1882[c] – 4d, 5b
S. tuba Kent, 1880– 1882[c] – 3b
****Acanthoecida Cavalier-Smith, 1997
*****Acanthoecidae Norris, 1965 *sensu* Nitsche et al., 2011
Acanthoeca spectabilis Ellis, 1930[c] – 3b
*****Stephanoecidae Leadbeater, 2011
Acanthocorbis camarensis Hara in Hara et al., 1996 – 2a, 3a, 3b, 6, 7
Stephanoeca apheles Thomsen, Buck and Chavez, 1991[c] – 3b, 6, 7
S. cupula (Leadbeater, 1972) Thomsen, 1988[c] – 3b, 5a
S. diplocostata Ellis, 1930[c] – 3b
S. supracostata Hara in Hara et al., 1996[c] – 6, 7
Volkanus costatus (Valkanov, 1970) Özdikimen, 2009[c] – 3d, 6, 7
DIAPHORETICKES Adl et al., 2012
SAR Burki et al., 2008
*Alveolata Cavalier-Smith, 1991
**Colpodellida Cavalier-Smith, 1993
***Colpodellaceae Adl et al., 2019
Colpodella sp. – 6
**Colponemida Cavalier-Smith 1993
***Colponemidia Tikhonenkov et al. 2014
Colponema marisrubri Mylnikov and Tikhonenkov, 2009 – 3c
*Rhizaria Cavalier-Smith, 2002
**Cercozoa Cavalier-Smith, 1998
***Thecofilosea Cavalier-Smith, 2003
****Cryomonadida Cavalier-Smith, 1993
*****Protaspidae Cavalier-Smith, 1993
Protaspa obliqua (Larsen and Patterson, 1990) Cavalier-Smith in Howe et al., 2011[c] – 6
P. tegere (Larsen and Patterson, 1990) Cavalier-Smith in Howe et al., 2011[c] – 6
P. verrucosa (Larsen and Patterson, 1990) Cavalier-Smith in Howe et al., 2011[c] – 7
***Imbricatea Cavalier-Smith, 2011
****Marimonadida Cavalier-Smith and Bass, 2011
Cyranomonas australis Lee, 2002[c] – 2a, 7
Cyranomonas sp. – 6
****Variglissida Cavalier-Smith, 2014
Clautriavia biflagellata Chantangsi and Leander, 2009 – 3a, 5b, 6, 7
****Silicofilosea Adl et al., 2005
*****Thaumatomonadida Shirkina, 1987
******Thaumatomonadidae Hollande, 1952
Thaumatomonas seravini Mylnikov and Karpov, 1993[c] – 5b
***Metromonadea Cavalier-Smith, 2007

Table 2 (*Continued*)

Metopion fluens Larsen and Patterson, 1990[c] – 2a, 2b, 3c, 4b, 5b, 5c, 6, 7, 8a
Metromonas grandis Larsen and Patterson, 1990[c] – 2a, 2b, 5b, 6
M. simplex (Griessmann, 1913) Larsen and Patterson, 1990[c] – 2a, 2b, 7
***Granofilosea Cavalier-Smith and Bass, 2009
****Massisteridae Cavalier-Smith, 1993
Massisteria marina Larsen and Pattersen, 1990[c] – 2a, 4d, 5b
***Glissomonadida Howe and Cavalier-Smith, 2009
****Dujardinidae Howe and Cavalier-Smith, 2011
****Allapsidae Howe and Cavalier-Smith, 2009
Allantion tachyploon Sandon, 1924[c] – 3a
Incertae sedis Cercozoa
Discocelis punctata Larsen and Patterson, 1990[c] – 5b, 7
*Stramenopiles Patterson, 1989
**Bigyra Cavalier-Smith, 1998
***Opalozoa Cavalier-Smith, 1991
****Bicosoecida Grasse, 1926
Bicosoeca gracilipes James-Clark, 1867[c] – 2b
B. maris Picken, 1841[c] – 1b, 8b
Caecitellus parvulus (Griessmann, 1913) Patterson et al., 1998[c] – 2a, 2e, 4d, 5c, 6, 7, 8b
Cafeteria ligulifera Larsen and Patterson, 1990[c] – 3a, 4a, 5b, 6
C. minuta (Ruinen, 1938) Larsen and Patterson, 1990[c] – 4d, 5c
C. roenbergensis Fenchel and Patterson, 1988[c] – 2a, 2b, 2c, 3a, 3b, 4a, 4b, 5a, 5b, 5c, 6, 7
Halocafeteria seosinensis Park et al., 2006 – 8b
Pseudobodo tremulans Griessmann, 1913[c] – 6
**Gyrista Cavalier-Smith, 1998
***Ochrophyta Cavalier-Smith, 1986
****Diatomista Derelle et al., 2016
*****Dictyochophyceae Silva, 1980
******Pedinellales Zimmermann et al., 1984
Actinomonas mirabilis Kent, 1880–1882[c] – 4d
Ciliophrys infusionum Cienkowski, 1876[c] – 5c, 6, 8b
Pteridomonas danica Patterson and Fenchel, 1985[c] – 3b, 3c, 4d, 5a
****Chrysista Cavalier-Smith, 1986
*****Chrysophyceae Pascher, 1914
******Paraphysomonadida Scoble and Cavalier-Smith, 2014
Clathromonas butcheri (Pennick and Clarke, 1972) Scoble and Cavalier-Smith, 2014[c] – 3a
Paraphysomonas foraminifera Lucas, 1967[c] – 3b, 6
Paraphysomonas sp. – 3d
Cryptista Adl et al., 2018
*Cryptophyceae Pascher, 1913
**Cyathomonadacea Pringsheim, 1944
Goniomonas pacifica Larsen and Patterson, 1990[c] – 2a, 3d, 6, 7
G. truncata (Fresenius, 1858) Stein, 1878[c] – 2a, 2d, 3b, 4d, 5c, 6, 7
Goniomonas sp. – 8a
Haptista Cavalier-Smith, 2003
*Centroplasthelida Febvre-Chevalier and Febvre, 1984
**Panacanthocystida Shishkin and Zlatogursky, 2018
****Acanthocystida Cavalier-Smith and von der Heyden, 2007
*****Chalarothoracina Hertwig and Lesser, 1874 *sensu* Cavalier-Smith, 2012
******Raphidocystidae Zlatogursky, 2018
Raphydocystis bruni (Penard, 1903) Zlatogursky, 2018 – 2a
**Pterocystida Cavalier-Smith and von der Heyden, 2007
***Raphidista Shishkin and Zlatogursky, 2018
****Choanocystidae Cavalier-Smith and von der Heyden, 2007
Choanocystis perpusilla (Petersen and Hansen, 1960) Siemensma, 1991[c] – 8b
***Pterista Shishkin and Zlatogursky, 2018
****Heterophryidae Poche, 1913
Heterophrys-like organism – 5c

Table 2 (*Continued*)

Incertae sedis Diaphoretickes
Telonemia Shalchian-Tabrizi, 2006
Telonema subtile Griessmann, 1913[c] – 2b, 6
Incertae sedis EUKARYA: EXCAVATES [Excavata Cavalier-Smith, 2002]
*Discoba Simpson, 2009
**Euglenozoa Cavalier-Smith, 1981
***Kinetoplastea Honigberg, 1963
Incertae sedis Kinetoplastea
Pseudophyllomitus apiculatus (Skuja, 1948) Lee, 2002 *sensu* Mylnikov, 1986[c] – 2a, 2b, 2d, 3a, 3b, 5b
****Metakinetoplastina Vickerman, 2004
*****Neobodonida Vickerman, 2004
Neobodo designis (Skuja, 1948) Moreira et al., 2004[c] – 2a, 2c, 2d, 3a, 3b, 4a, 4b, 5b, 6, 7, 8a
N. saliens (Larsen and Patterson, 1990) Moreira et al., 2004[c] – 2a, 3a, 4b, 5b, 6, 8a
Rhynchomonas nasuta (Stokes, 1888) Klebs, 1893[c] – 2a, 2c, 2d, 3a, 3b, 3c, 3d, 4a, 4b, 4c, 4d, 5b, 5c, 6, 7, 8a, 8b
Rhynchobodo simius Patterson and Simpson, 1996[c] – 6
***Euglenida Butschli, 1884
****Heteronematina Leedale, 1967
Dinema platysomum (Skuja, 1939) Lee and Patterson, 2000[c] – 6
Lentomonas azurina (Patterson and Simpson, 1996) Cavalier-Smith, 2016[c] – 2a, 2b, 5b, 5c, 6, 7
L. corrugata (Larsen and Patterson, 1990) Cavalier- Smith, 2016[c] – 2a, 6, 7
Petalomonas cantuscygni Cann and Pennick, 1986 – 1a, 1b, 3a
P. minor Larsen and Patterson, 1990[c] – 1b, 2a, 3a, 6, 7
P. minuta Hollande, 1942[c] – 5a, 5b, 6, 7
P. ornata Skvortzov, 1957[c] – 3d
P. poosilla (Skuja, 1948) Larsen and Patterson, 1990[c] – 2a, 3a, 3b, 4d, 5a, 5b, 6, 7, 8b
Petalomonas sp. – 3c
Ploeotia adhaerens Larsen and Patterson, 1990[c] – 6, 7
P. aff. *costata* (Triemer, 1986) Farmer and Triemer, 1988[c] – 7
P. discoides Larsen and Patterson, 1990[c] – 5b, 7
P. oblonga Larsen and Patterson, 1990[c] – 2a, 8a
P. punctata Larsen and Patterson, 1990 – 3a
P. robusta Larsen and Patterson, 1990 – 3b
Ploeotia sp. 1 – 2a, 5a, 5b, 5c
Ploeotia sp. 2 – 2a, 2b, 3c, 5a, 5b, 6, 8a
***Diplonemea Cavalier-Smith, 1993
****Diplonemidae Cavalier-Smith, 1993
Diplonema ambulator Larsen and Patterson, 1990[c] – 2c, 5b
Rhynchopus amitus Skuja, 1948[c] – 2a, 8b
**Heterolobosea Page and Blanton, 1985
***Tetramitia Cavalier-Smith, 1993
****Eutetramitia Hanousková et al., 2018
*****Percolomonadidae Cavalier-Smith, 2008
Percolomonas cosmopolitus (Ruinen, 1938) Fenchel and Patterson, 1986[c] – 1a, 7
P. denhami Tong, 1997[c] – 1b, 2a, 2c, 5b, 6
P. similis Lee et al., 2003[c] – 1a, 2a
Incertae sedis Eukarya: "CRuMs"
*"CRuMs" (Brown et al., 2018)
Glissandra innuerende Patterson and Simpson, 1996[c] – 6
**Mantamonas* Cavalier-Smith and Glücksman, 2011
Mantamonas plastica Clücksman and Cavalier-Smith in Glücksman et al., 2011 – 3a, 6, 7
Incertae sedis Eukarya: Ancyromonadida Cavalier-Smith, 1998
Ancyromonas sigmoides (Kent, 1880–1882) Heiss et al., 2010[c] – 2a, 3a, 3d, 5b, 6, 7, 8b

Table 2 (*Continued*)

Other *incertae sedis* EUKARYA
"*Heterochromonas opaca*" *sensu* Lee and Patterson, 2000 – 6
"Protist 1" – 7
"Protist 2" – 7
"Protist 3" – 3c
"Protist 4" – 2d, 6
"Protist 5" – 2a

See Table 1 for sampled biotopes (1a – 8b) description.
"c" – presumably cosmopolitan morphospecies recorded from all hemispheres (North, South, West, and East) and from equatorial, tropical or subtropical, temperate, and polar regions.
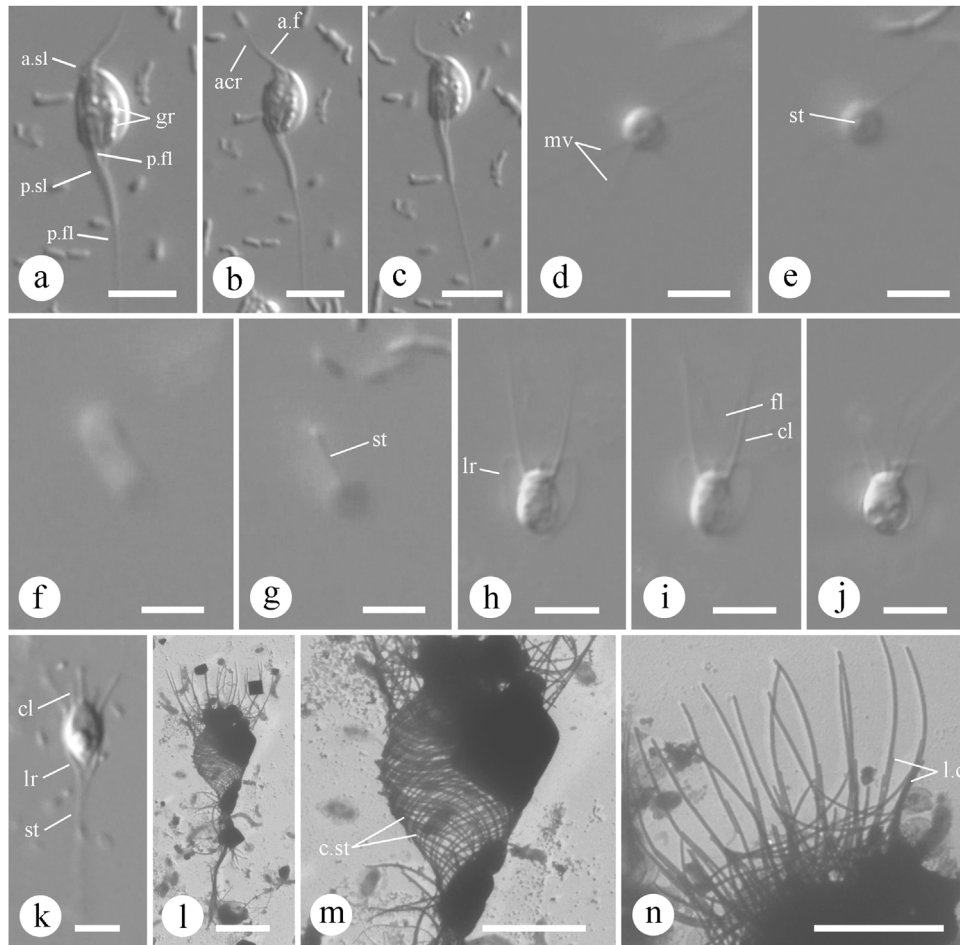

Fig. 1. Morphology of observed heterotrophic flagellates (a–k – DIC, l–n – TEM): a–c – *Thecamonas* sp.; d–g – *Ministeria vibrans* (d, e – non-moving cell, f–g – moving cell); h–j – *Salpingoeca pyxidium*; k–n – *Acanthoeca spectabilis* (k – living cell, l – whole lorica; m – posterior chamber of the lorica; n – anterior chamber of the lorica). Abbreviations: acr – acronema; a.f – anterior flagellum; a.sl – anterior sleeve; cl – collar; c.st – costal strips; fl – flagellum; gr – granules; lr – lorica; l.c – longitudinal costa; mv – microvilli; p.fl – posterior flagellum; p.sl – posterior sleeve; st – stalk. Scale bars: 5 μm.

for biflagellate *Amastigomonas*-like protists whose anterior flagellum and dorsal cell body surface is covered with a pliable organic theca. We assigned specimens to the genus *Thecamonas* due to the presence of moderately rigid cell covering (probably, theca). Our organism differs from *Manchomonas* species by the presence of acronematic flagella; one long posterior flagellum that extends beyond the ventral groove, and a small and weakly noticeable triangular sleeve. We didn't observe multicellular plasmodia with incomplete cytokinesis, as seen in *Multimonas* species, in Petri dishes over the course of 10 days observation. Observed cells differed from *Podomonas* species by the absence of multiform finger-like, lamellar or reticulose pseudopodia. Ultrastructural and molecular data are needed for confir-

mation of the position of this species within the genus *Thecamonas*.

Observed species differs from all known *Amastigomonas*-like species, except *Podomonas* (=*Amastigomonas*) *klosteris* (Arndt and Mylnikov in Mylnikov, 1999) Cavalier-Smith in Cavalier-Smith and Chao, 2010, because of the presence of long posterior sleeve surrounding the posterior flagellum. However, *Podomonas klosteris* is characterized by a larger cell body (length 12–20 μm) with a posterior sleeve that is shorter relative to the cell (about one-quarter of the cell body length), and presence of pseudopodia emerging from the ventral groove (Mylnikov 1999).

*Opisthokonta Cavalier-Smith, 1987
**Holozoa Lang et al., 2002
***Filasterea Shalchian-Tabrizi et al., 2008
Ministeria vibrans Tong, 1997 (Fig. 1d–g)

Observations: The spherical cell body is 2.5–3.0 μm in diameter, with 10–15 radial thin microvilli (arms) 4–5 μm in length. Cells attach to the substrate with a stalk (Fig. 1g) and make fast pendulum movements with a small amplitude (Fig. 1f). Sometimes cells stop moving for a while. Flagellates feed on bacteria, predominantly rod-like, that adhere to the surface of the cell body. Nine cells observed in LM.

Remarks: Mylnikov and coauthors (Mylnikov et al. 2019) showed that *Ministeria* arms are supported by bundles of axial microfilaments that correspond in structure to the microvilli surrounding the collar of choanoflagellates, and which can be drawn into the cell body. This species differs from another member of the genus, *M. marisola* Patterson et al., 1998, by having a larger number of radial filopodia, as well as attachment to a substrate with a stalk and pendulum movements (Tong, 1997b).

Distribution: Marine waters of UK (Tong 1994), Australia (Lee et al. 2003), Black Sea (Prokina et al. 2018), Baltic Sea (Mylnikov et al. 2019). Fresh waters of European part of Russia, Kostroma Region (unpublished), Hungary (Kiss et al. 2009). Hypersaline inland waters of Southern Chile, Tierra del Fuego (unpublished).

***Choanoflagellata Kent, 1880–1882
****Craspedida Cavalier-Smith, 1997
*****Salpingoecidae Kent, 1880–1882 *sensu* Nitsche et al., 2011
Salpingoeca pyxidium Kent, 1880–1882 (Fig. 1h–j)

Observations: The cell is located in an organic lorica, that is heart-shaped in outline: narrowed basal part and broad anterior part. Closer to the mouth of the lorica, its edges curved towards the cell and forming a small indentation. Lorica length is 5–6 μm, width at the expanded anterior part is 4.5–5.5 μm. Cells obovoid, 4–5 μm long, 3.0–3.5 μm wide, with slightly expanded basal part and truncated anterior end. Collar length is 1–2 times longer than the cell, located mostly outside the lorica. The single flagellum is slightly longer than the cell. Nucleus located medially. Several food vacuoles located posteriorly. The stalk wasn't observed, cells attached

to the substrate with the basal part of the lorica. Three cells observed in LM.

Remarks: Some authors described the lorica as almost circular in outline (Francé 1897; Lemmermann 1910; Starmach 1968; Tikhonenkov et al., 2008a,b; Zhukov and Karpov 1985), although Kent in the original description (Kent, 1880) pointed out the heart-shaped morphology of the lorica. The morphology of the cells we observed corresponded to the morphology described by Kent. Sizes of cells are in agreement with previous descriptions, except the length of the flagellum in observed cells is almost equal to cell length, while many authors noted flagellum length is 2.5 times longer than the cell.

Distribution: Fresh waters of European part of Russia (Kopylov et al. 2015; Tikhonenkov and Mazei 2007), U.K. (Kent 1880), Hungary (Francé 1897), Poland (Starmach 1968). Black Sea (Tikhonenkov 2006), Pechora Sea (Mazei and Tikhonenkov, 2006; Tikhonenkov 2006).

****Acanthoecida Cavalier-Smith, 1997
*****Acanthoecidae Norris, 1965 *sensu* Nitsche et al., 2011
Acanthoeca spectabilis Ellis, 1930 (Fig. 1k–n)

Observations: The living cell measured was 8.0 × 4.5 μm, with a rounded anterior end and tapered posterior end. Cell located in conical lorica 12.5–16.0 μm in length. Lorica consists of the anterior chamber (Fig. 1n) and the posterior chamber (Fig. 1m). The anterior chamber is 4.5–6.5 μm in length, 7.5–10.5 μm in diameter, consist of 15–16 longitudinal costae, each of them includes two rod-like costal strips. The posterior chamber is 8–9 μm in length, 5.5–6.0 μm in diameter, and consist of numerous helically twisted costae. Lorica attached to the substrate with a long and thick stalk, 11–20 μm in length, 0.4–0.6 μm in diameter. The base of stalk expanded. Stalk consists of many rod-like costal strips oriented parallel to the longitudinal axis of the stalk and tightly adjacent to each other. At the base of the posterior chamber, the elements gradually twist into a spiral. Descriptions based on observation of one living cell in LM and six loricae in TEM.

Remarks: Morphology of observed lorica corresponds with previous descriptions, however, some authors recorded another number of longitudinal costae: 10–16 (Leadbeater 1972), 13–15 (Leadbeater et al. 2008), 14 costae in original descriptions (Norris 1965). This species differs from the second common species of the genus, *A. brevipoda* Ellis, 1930, by the presence of a stalk.

Distribution: Marine waters of U.K. (Leadbeater and Morton 1974), Denmark (Thomsen 1973), Norway (Leadbeater 1972), USA (Norris 1965), Australia (Lee 2015; Lee et al. 2003; Tong 1997c; Tong et al. 1998), Taiwan (Hara et al. 1997), Antarctica (Marchant and Perrin 1990), Baltic Sea (Vørs 1992). Hypersaline waters of Australia (Al-Qassab et al. 2002).

*****Stephanoecidae Leadbeater, 2011
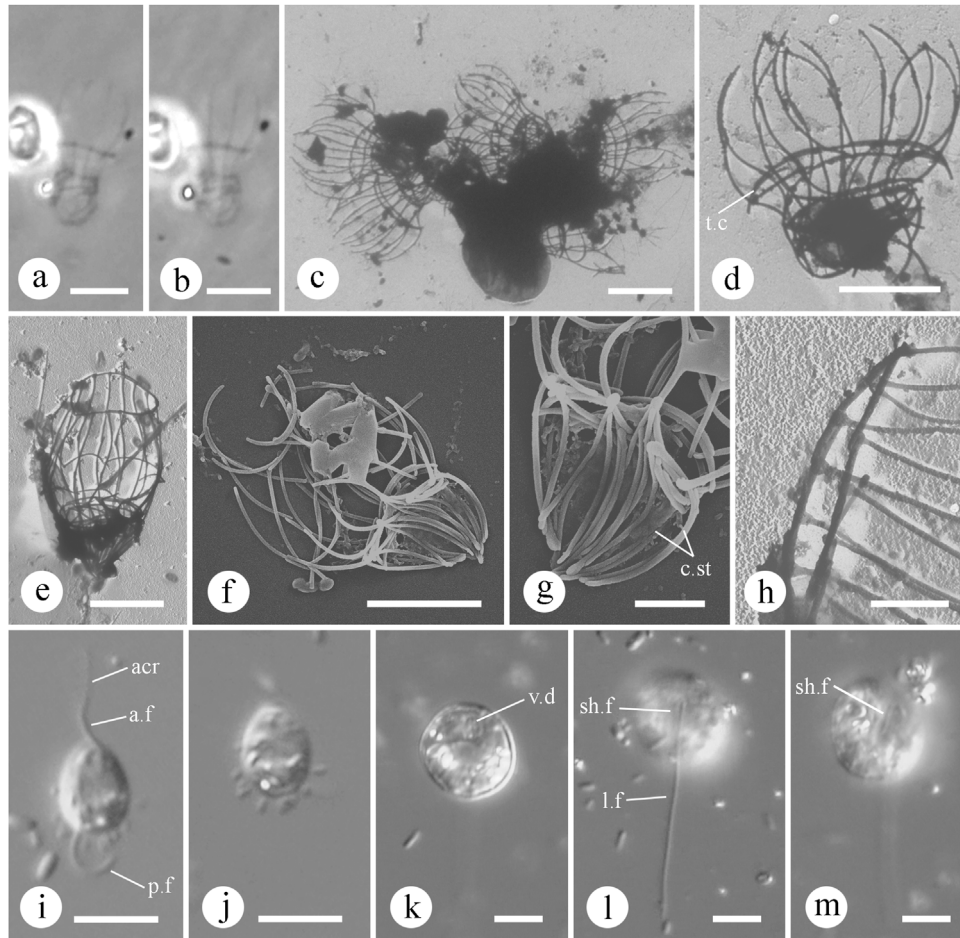***Acanthocorbis camarensis*** Hara in Hara et al., 1996 (Fig. 2a–d)

Fig. 2. Morphology of observed heterotrophic flagellates (a, b – PhC, c–e, h – TEM, f, g – SEM, i–m – DIC): a–d – *Acanthocorbis camarensis* (a, b – living cell, c, d – loricae); e–h – *Stephanoeca supracostata* (e, f – whole lorica, g – posterior chamber, h – mouth of the lorica); i, j – *Cyranomonas australis* (i – flagella in focus, j – attached particles in focus); k–m – *Clautriavia biflagellatta*. Abbreviations: acr – acronema; a.f – anterior flagellum; c.st – costae strips; l.f – long flagellum; p.f – posterior flagellum; sh.f – short flagellum; t.c – transverse costa; v.d – ventral depression. Scale bars: a–f, i–m – 5 μm; g, h – 2 μm.

Observations: Lorica is 10.5–14.5 μm in length, consists of two chambers. The anterior chamber is 6.6–9.2 μm in length, 10.3–12.0 μm in diameter, expands in the central part, and tapers slightly towards the mouth. Consist of 12–14 longitudinal costae and one double transverse costa (or two transverse costae adjacent). Longitudinal costae consist of three rod-like costal strips, 3.4–4.9 μm in length, 0.15–0.20 μm in diameter. Transverse costa of the anterior chamber located closer to the basal part of lorica, consist of many rod-like costal strips, overlapping each other. Posterior chamber bell-shaped, often obscured by protoplast, 3.8–6.3 μm in length, 5.2–5.7 μm in diameter, consist of several transverse and helical costal strips. The number of longitudinal costae of the posterior chamber, probably, equal to the number of longitudinal costae of the anterior chamber. Description based on records of one living cell in LM, and eleven loricae in TEM.

Remarks: Morphology of observed lorica corresponds with previous descriptions, however, Thomsen and Østergaard (2019) indicated up to 18 longitudinal ribs. We recorded only 12–14 (often 14) ribs, which matches the original descrip-

tion, 10–14 longitudinal ribs in Hara et al. (1996). This species close resembles *A. apoda* (Leadbeater, 1972) Hara and Takahashi, 1984 by the presence of a double transverse rib, absence of stalk, as well as the presence of ёmpty spaceBetween the transverse ribs of anterior and posterior chambers (Leadbeater 1972). However, *A. camarensis* can be distinguished by the shorter distance between anterior transverse costa and the lorica waist (less than the length of one longitudinal costal strip), and the presence of additional helical costal elements in the posterior chamber (Thomsen and Østergaard 2019).

Distribution: Marine water of Hawaii, Thailand, Taiwan (Hara et al. 1996; Thomsen and Østergaard 2019), Australia (Lee et al., 2003).

Stephanoeca supracostata Hara in Hara et al., 1996 (Fig. 2e–h)

Observations: Living cells were not observed. Cell surrounded by basket-like lorica, consisting of siliceous costa. Lorica length (according to TEM and SEM whole mount) is 10.5–14.0 μm, diameter in the broad middle part is 7.5–8.0

μm. Lorica consists of anterior and posterior chambers. The anterior chamber is 7.0–9.5 μm in length, includes 12–14 longitudinal costae and three transverse costae. Longitudinal costae consist of two rod-like costal strips wih flattened tips, connected to each other with a slight overlap. Anterior transverse costae located at the lorica mouth are 5–6 μm in diameter. The second transverse costa is 4.5–5.5 μm in diameter, located on the broadest part of lorica, near the overlap of longitudinal costal strips. The third transverse costa is located at the junction of anterior and posterior chambers. The posterior chamber is 4.0–4.5 μm in length, tapers conically to the basal end, and consists of numerous longitudinal costal strips with both tips flattened. Observation is based on a description of four loricae in TEM and one lorica in SEM.

Remarks: Morphology and size of observed cells correspond with previous descriptions: lorica length is 9–18 μm (Hara et al. 1996; Thomsen and Østergaard 2019); lorica width is 4.5–10 μm (Hara et al. 1996); the number of longitudinal costae is 7–20 (Hara et al. 1996; Lee et al. 2003). The morphology of the observed loricae is very similar to *S. elegans* (Norris, 1965) Throndsen, 1974. However, specimens studied here can be distinguished by the presence of a third middle transverse costa where the lorica has its maximum diameter, as well as the conically tapered posterior chamber. Other species of *Stephanoeca* have more than three transverse costae.

Distribution: Marine waters of Australia (Lee et al. 2003), Taiwan, Japan (Hara et al. 1996), Thailand, Denmark (Thomsen and Østergaard 2019), U.K. (Tong 1997a).

DIAPHORETICKES Adl et al., 2012
Sar Burki et al., 2008
*Rhizaria Cavalier-Smith, 2002
**Cercozoa Cavalier-Smith, 1998
***Imbricatea Cavalier-Smith, 2011
****Marimonadida Cavalier-Smith and Bass, 2011
Cyranomonas australis Lee, 2002 (Fig. 2i, j)

Observations: Cells are ovoid in outline, with a slightly narrowed anterior end, and dorso-ventrally flattened. Cell length is 4.5–5.5 μm, width is 3.5–4.5 μm. Flagella insert subapically from small ventral depression. The anterior flagellum is 1.0–1.2 times longer than the cell, acronematic, directed anterior-lateraly and makes flapping movements. The posterior flagellum is twice as long as the cell and trails behind the cell. Numerous particles attached to the posterior part of the cell (Fig. 2j). Nucleus located anteriorly. Seven cells were observed in LM.

Remarks: Cell sizes are similar to those described previously (Aydin and Lee 2012; Lee 2002; Tikhonenkov 2009), however, particles attached to the posterior part of the cell body were not previously indicated. Also, the presence of an acroneme on the anterior flagellum was not previously noted (Aydin and Lee 2012; Lee 2002; Tikhonenkov 2009).

Distribution: Red Sea (Tikhonenkov 2009), White Sea ([as Flagellata sp. 1] Tikhonenkov et al. 2006; Tikhonenkov and Mazei 2013), marine waters of Turkey (Aydin and Lee 2012), South Korea, and Australia (Lee 2002).

****Variglissida Cavalier-Smith, 2014
Clautriavia biflagellata Chantangsi and Leander, 2009 (Fig. 2k–m)

Observations: Cells are circular in outline, dorso-ventrally flattened, with slightly concave ventral surface and anterior-medial depression. The cell diameter is 9–13 μm. Two unequal non-acronematic flagella emerge from ventral depression and both directed posteriorly. The long flagellum is 2.0–2.3 times longer than the cell and trails behind the cell. The short flagellum is 2.5–3.0 μm in length, immobile, and slightly thinner than the long flagellum. It is weakly visible during normal cell movement (Fig. 2l) and can be noticed when cells rise above the substrate (Fig. 2m). Pseudopodia were not observed. Four cells were observed in LM.

Remarks: Morphology of observed cells corresponds with the original description, while being at the smaller end of the size range observed in the type material (Chantangsi and Leander 2010). This species is distinguishable from other species of the genus *Clautriavia* by the presence of a second short flagellum and the circular cell outline. Feeds on algae through the ventral side of the cell (Chantangsi and Leander 2010).

Distribution: Marine waters of British Columbia, Canada (Chantangsi and Leander 2010).

****Silicofilosea Adl et al., 2005
*****Thaumatomonadida Shirkina, 1987
******Thaumatomonadidae Hollande, 1952
Thaumatomonas seravini Mylnikov and Karpov, 1993 (Fig. 3a–c)

Observations. Living cells were not observed. According to the TEM whole mount, protoplast is ovate, 6.5–7.5 μm long, and 4.5–6.0 μm wide. The long flagellum is 20–23 μm in length, short flagellum not observed. Cells covered by numerous siliceous scales of one type. Scales consist of two triangular plates, connected to each other with three hollow cylindrical struts at the corners (0.2–0.3 μm in length). Sides of plates are about 0.5–0.6 μm in length. Proximal plate textureless, with more rounded corners. Distal plate with gently curved and more pointed corners and a row of perforations along the margin. A large oval hole located in each corner. Observations based on the description of scales of five cells in TEM.

Remarks: This species is common in freshwater, but is rare in marine waters (see distribution). Our observations correspond with previous findings, except that some authors describe larger cell sizes: length is 8.4–15.0 μm, width is 5.0–13 μm (Mylnikov and Karpov 1993; Mylnikov et al. 2002, 2006; Prokina et al. 2017; Tikhonenkov 2006). Cell protoplasts could shrink when dried. The size and morphology of observed scales are similar to described previously. This species is quite similar to *T. hindoni* (Nicholls, 2013) Scoble and Cavalier-Smith, 2014, described from fresh waters of Canada (Nicholls 2013). *T. seravini* differs from *T. hindoni* by larger perforations along plate margins, the presence of a large oval hole at each corner of the distal plate,
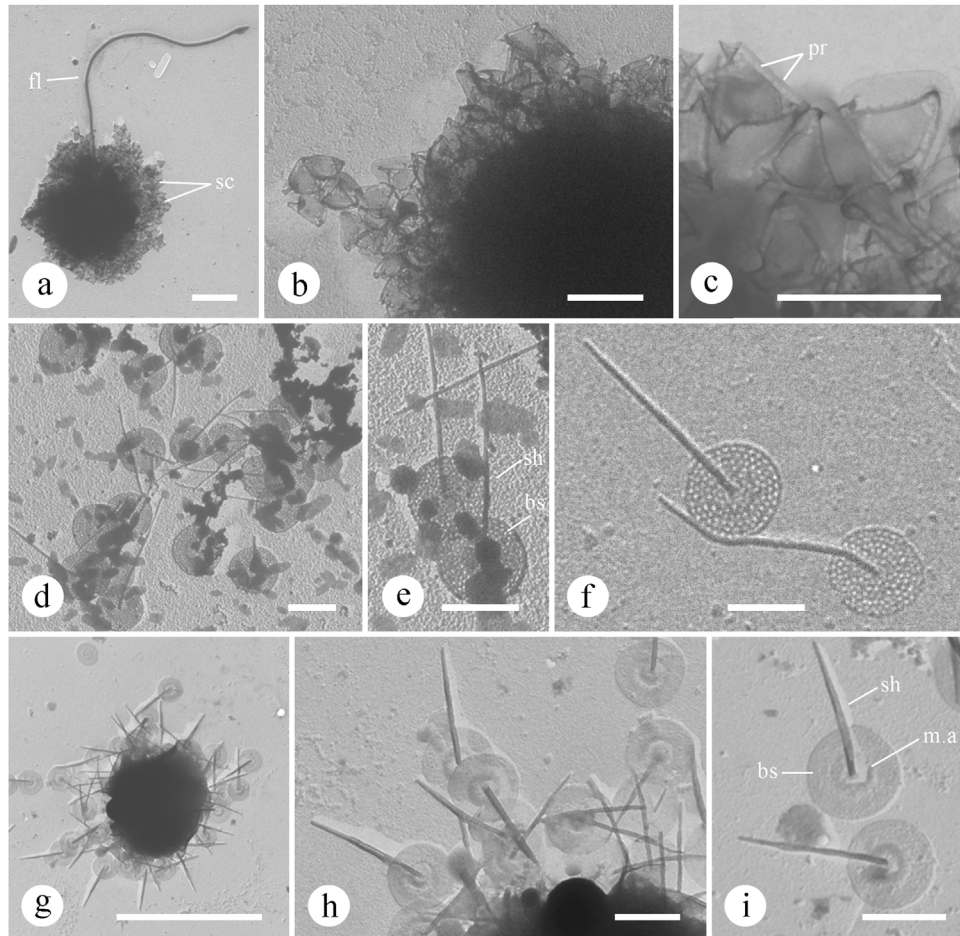
Fig. 3. Morphology of observed heterotrophic flagellates (TEM): a–c – *Thaumatomonas seravini*; d–f – *Paraphysomonas foraminifera*; g–i – *Paraphysomonas* sp. Abbreviations: bs – base of spine scale; fl – flagellum; m.a – middle annulus; sc – scales; sh – shaft; pr – perforations. Scale bars: a, g – 5 μm; b, c, d–f, h, i – 1 μm.

and more gently curved corners (Mylnikov and Karpov 1993; Nicholls 2013; Scoble and Cavalier-Smith 2014b). *T. zhukovi* Mylnikov, 2003 has similar scales, but is distinguished by the presence of two types of scales – oval and triangular. However, Scoble and Cavalier-Smith (2014b) studied the morphology and 18S rRNA sequences of several clones of this species and found that it can be polymorphic with two or one types of scales (either oval or triangular). These species can thus not be distinguished with certainty from scale morphology only. Their studies also have shown that both species are closely related.

Distribution: Freshwaters of European part of Russia (Kopylov et al. 2015; Mylnikov and Karpov 1993; Mylnikov et al. 2002, 2006; Prokina et al. 2017; Tikhonenkov 2006), Mongolia (Kopylov et al. 2006), Chile (Prokina and Mylnikov 2018), China (Tikhonenkov et al. 2012). Soils of China (Tikhonenkov et al. 2012). White Sea (Tikhonenkov 2006; Tikhonenkov and Mazei 2013).

*Stramenopiles Patterson, 1989
**Gyrista Cavalier-Smith, 1998
***Ochrophyta Cavalier-Smith, 1986
****Chrysista Cavalier-Smith, 1986
*****Chrysophyceae Pascher, 1914
******Paraphysomonadida Scoble and Cavalier-Smith, 2014

***Paraphysomonas foraminifera* Lucas, 1967 (Fig. 3d–f)**

Observations: Living cells were not observed. Cells covered by siliceous hobnail-like scales of one type. Scales consist of a circular flattened base and cylindrical shaft. The base is 0.7–1.2 μm in diameter, perforated with 10 or more concentric rows of round holes, which are not always clearly visible. The shaft is 1.9–3.0 μm in length, 0.05–0.1 μm in diameter, with a sharp end. Several single scales and scale aggregations of two cells observed in TEM.

Remarks: Many authors described smaller length of the shaft, 0.45–1.3 μm (Bergesch et al. 2008; Lukas 1967; Tong 1997b, c; Vørs 1993a); 7–8 concentric rows of holes on base (LeRoi and Hallegraeff 2006; Tong 1997c; Tong et al. 1998), as well as the presence of middle dense annulus (LeRoi and Hallegraeff 2006; Tong 1997c), and "shoulders" – sharp narrowing of the diameter of the shaft in the middle of its length (Tong 1997c). Nicholls (1981) described several scales from freshwater in Canada, under the name *P. foraminifera*. However, these scales probably belong to sev-

eral *Paraphysomonas* or *Clathromonas* species because of significant differences: the presence of perforations on the basal part of the shaft, as well as the reduced shaft on some scales, the larger size of scales (base up to 2.5 μm). Several scales (Nicholls 1981: Fig. 16–18, p. 134) are very similar to scales of *Clathromonas takahashii* (Cronberg and Kristiansen, 1981) Scoble and Cavalier-Smith, 2014 and one scale (Nicholls 1981: Fig. 19, p. 134) is very similar to scales of *Clathromonas elegantissima* (Kling and Kristiansen, 1983) Scoble and Cavalier-Smith, 2014.

Distribution: Baltic Sea (Vørs 1992), Mediterranean Sea (Throndsen and Zingone 1994), marine waters of Denmark (Thomsen, 1975), U.K. (Tong 1997b), Croatia (Leadbeater 1974), Brazil (Bergesch et al. 2008), Canada (Smith and Hobson 1994), Australia (LeRoi and Hallegraeff 2006; Tong 1997c; Tong et al. 1998), and Greenland (Vørs 1993a).

Paraphysomonas sp. (Fig. 3g–i)

Observations: Living cells were not observed. Cells are covered by siliceous hobnail-like scales of one type. Scales consist of a circular flattened base and cylindrical shaft. The base is 0.6–0.9 μm in diameter, with a dense middle annulus. The shaft is 1.3–1.9 μm in length, 0.08–0.1 μm in diameter, conically pointed into a rounded tip. Scales of two cells observed in TEM.

Remarks: Scales of this species are most similar to scales of *P. imperforata* Lukas, 1967 as evidenced by a small length of the shaft, base plate with middle annulus, and lacking dense margins. But observed scales are differentiated by a noticeably thinner shaft without conical tapering. Other species with middle annulus on the base plate (*P. acuminata acuminata* Scoble and Cavalier-Smith, 2014; *P. acuminata cuspidata* Scoble and Cavalier-Smith, 2014; *P.mikadiforma* Scoble and Cavalier-Smith, 2014; *P. hebetispina hebetispina* Scoble and Cavalier-Smith, 2014) differ due to a very long shaft relative to base plate diameter (Scoble and Cavalier-Smith 2014a).

Haptista Cavalier-Smith, 2003
*Centroplasthelida Febvre-Chevalier and Febvre, 1984
**Panacanthocystida Shıshkin and Zlatogursky, 2018
****Acanthocystida Cavalier-Smith and von der Heyden, 2007
*****Chalarothoracina Hertwig and Lesser, 1874 *sensu* Cavalier-Smith, 2012
******Raphidocystidae Zlatogursky, 2018

Raphidocystis bruni (Penard, 1903) Zlatogursky, 2018 (Fig. 4a–c)

Synonyms: *Raphidiophrys bruni* Penard, 1903; *Polyplacocystis bruni* (Penard, 1903) Mikrjukov, 1999.

Observations: Cells covered by tangentially-oriented elongated-oval plate scales of one type. Scales are 5.9–8.1 μm in length, slightly widened in the central part (0.5–0.8 μm) and gradually tapering towards both ends (where they are 0.3–0.4 μm), at the most narrow point forming circular poles 0.5–0.6 μm in width. The length to width ratio is 8.2–13.8. The proximal surface of scales is smooth, without a longitudinal rib. The hollow marginal rim is 0.1–0.2 μm

in diameter in the middle and extends to the poles. Scales of one cell were observed in SEM.

Remarks: This species can be distinguished from other species of *Raphidocystis* due to elongated shape and circular poles, the lack of reticular structure on the proximal surface of scales, and the absence of spine scales.

Distribution: Fresh waters of Crimea (Mikrjukov 1999). Black Sea (Mikrjukov 1999), Tasman Sea (Mikrjukov 2002).
**Pterocystida Cavalier-Smith and von der Heyden, 2007
***Raphidista Shıshkin and Zlatogursky, 2018
****Choanocystidae Cavalier-Smith and von der Heyden, 2007

Choanocystis perpusilla (Petersen and Hansen, 1960) Siemensma, 1991 (Fig. 4d–g)

Synonyms: *Acanthocystis perpusilla* Petersen and Hansen, 1960; *Choanocystis cordiformis* ssp. *parvula* Dürrschmidt, 1987.

Observations: Cells covered by spine and plate scales. Radial spine scales consist of a hollow cylindrical shaft, asymmetrically extending from the heart-shaped flattened base. Shaft is slightly curved, 2.7–8.0 μm in length, 0.15–0.2 μm in diameter, and the distal tip divides into two furcae. The base is 1.1–1.7 μm in diameter, with a small notch. Tangential oval plate scales are 1.5–2.5 × 1.5–2.0 μm, with an axial ridge located inside medial depression. We observed scales of four cells in TEM.

Remarks: The size of observed scales corresponds with scales of subspecies *Ch. cordiformis parvula*, described by Dürrschmidt, 1987. Most often a shorter length of spine scales is indicated in the literature, 1.4–5.6 μm (Croome et al. 1987; Dürrschmidt 1985; Shatilovich et al. 2010), however, there are records with a length 8–10 μm (Leonov and Mylnikov 2012). Also, we previously described textureless plate scales, without an axial ridge (Prokina and Mylnikov 2019).

Distribution: Freshwater of European part of Russia (Leonov and Mylnikov 2012), Estonia (Mikrjukov 1993), Chile (Dürrschmidt 1985; Prokina and Mylnikov 2019), Sri Lanka ([as *Acanthocystis cordiformis parvula*] Dürrschmidt 1987), Vietnam (Prokina et al. 2019). Soil of East Siberia, Russia (Shatilovich et al. 2010). Marine waters of Antarctica (Croome et al. 1987). Baltic Sea (Vørs 1992). Saline inland waters of European part of Russia (Ermolenko and Plotnikov 2013).
***Pterista Shıshkin and Zlatogursky, 2018
****Heterophryidae Poche, 1913

Heterophrys-like organism (Fig. 4h–j)

Observations: Cells covered by radial organic fusiform spicules 5.2–11.5 μm in length, 0.05–0.1 μm in wide. Spicules are slightly flattened and spirally twisted along the longitudinal axis, and both tips are pointed. Basal part of spicules immersed in mucopolysaccharide capsule that is sometimes visible in TEM, as are axopodia. Spicules of five cells were observed in TEM.

Remarks: Centrohelids with spindle-shaped organic spicules have previously been found from various freshwater and marine habitats around the world, and have been
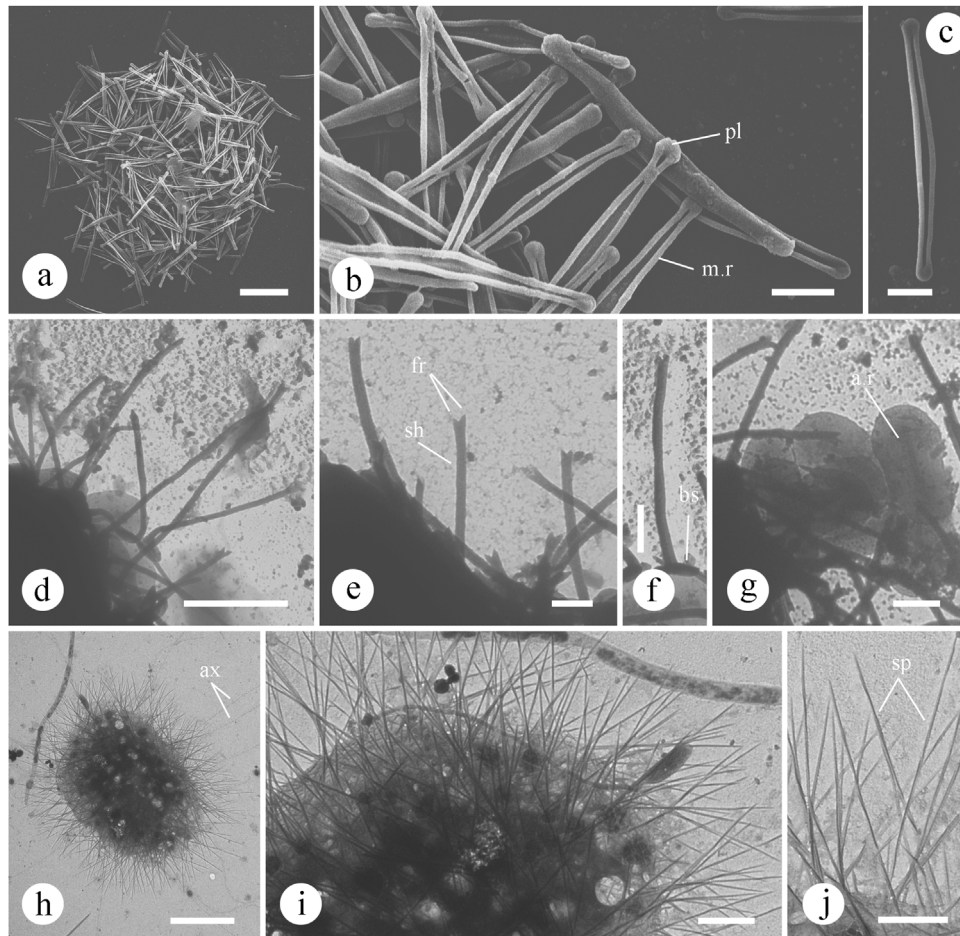
Fig. 4. Morphology of observed scales of centrohelid heliozoans (a–c – SEM; d–j – TEM): a–c – *Raphidocystis bruni* (a, b – scale aggregation of whole cell; c – single scale); d–g – *Choanocystis perpusilla* (d, e – scale aggregation of whole cell; f – spine scale; g – plate scales); h–j – *Heterophrys*-like organism. Abbreviations: a.r – axial ridge; ax – axopodia; bs – base of scale; fr – furcae; m.r – marginal rim; pl – poles of scales; sh – shaft; sp – spicules. Scale bars: a, d, h – 5 μm; b, c, e–g, i, j – 1 μm.

identified as species of genera *Heterophrys*, *Marophrys*, and *Sphaerastrum* (Cavalier-Smith and von der Heyden 2007; Mikrjukov 2002). However, recent studies have revealed close relations between spicule-bearing *Heterophrys*-like organisms and siliceous scales-bearing species both by molecular data (18S rDNA gene similarity of *Polyplacocystis* and *Heterophrys*), and by morphological data: intermediate polymorphic stages of the life cycle of *Raphidiophrys*, bearing both siliceous scales and organic spicules (Zlatogursky 2016; Zlatogursky et al. 2018). Based on these observations, Zlatogursky with coauthors (Zlatogursky et al. 2018) suggested dimorphism of centrohelid life cycle with two stages, one with siliceous scales and another with organic spicules. Thus, the spicules of the cells we studied may be the stage of the life cycle of one or more scale-bearing centrohelid species.

Incertae sedis EUKARYA: EXCAVATES [Excavata Cavalier-Smith, 2002, emend. Simpson, 2003]

   *Discoba Simpson, 2009

   **Euglenozoa Cavalier-Smith, 1981

   ***Euglenida Butschli, 1884

   ****Heteronematina Leedale, 1967

   ***Petalomonas cantuscygni* Cann and Pennick, 1986 (Fig. 5a–c)

Observations: Cells are ovoid in outline, anterior end slightly narrowed with well-marked openings of flagellar pocket (Fig. 5a). Cell length is 9.5–14.0 μm, width is 6–9 μm. The single flagellum is about the cell length or slightly longer, directed anteriorly. Dorsal side of the cell with 6 gentle ridges (Fig. 5b), ventral side with two slightly noticeable ridges. Flagellar pocket at the right side of the cell (viewed from the dorsal side). Nucleus located in the anterior part of the cell. Observations based on four cells found in LM.

Remarks: Our observations completely correspond with previous descriptions of this species. Cell length is 16 μm (Larsen and Patterson 1990) and 9.2–15.5 μm in the original description (Cann and Pennick 1986). The other species of the genus *Petalomonas* all have oval cell outlines (*P. poosilla* (Skuja, 1948) Larsen and Patterson, 1990; *P. ingosus* Lee and Patterson, 2000; *P. labrum* Lee and Patterson, 2000; *P. minor* Larsen and Patterson, 1990; *P. minuta* Hollande, 1942; *P. tricarinata* Skuja, 1939; *P. vigatus* Lee and Patterson, 2000),
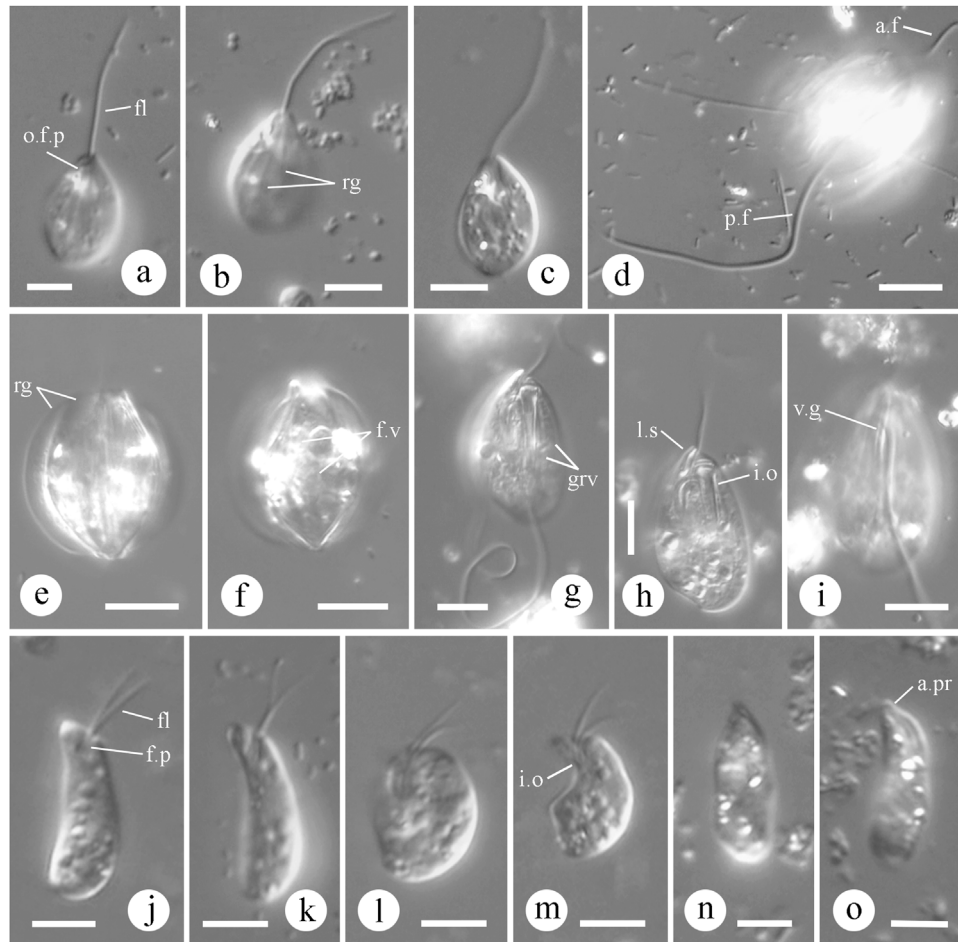
Fig. 5. Morphology of observed heterotrophic flagellates (DIC): a–c – *Petalomonas cantuscygni*; d–f – *Ploeotia adhaerens*; g–i – *P. robusta*; j–m – *Diplonema ambulator*; n, o – *Rhynchopus amitus*. Abbreviations: a.f – anterior flagellum; a.pr – apical protrusion (papilla); fl – flagellum; f.p – flagellar pocket; f.v – food vacuole; grv – grooves; i.o – ingestion organelle; l.s – lip-like structure; o.f.p – openings of flagellar pocket; p.f – posterior flagellum; rg – ridges; v.g – ventral groove. Scale bars: a–c, j–o – 5 μm; d–i – 10 μm.

and differ in number or noticeability of dorsal and ventral ridges, or by the presence of a smooth cell surface.

Distribution: Marine waters of U.K. (Cann and Pennick 1986), Brazil (Larsen and Patterson 1990).

Ploeotia adhaerens Larsen and Patterson, 1990 (Fig. 5d–f)

Observations: Cells not flattened, almost circular in outline, with four prominent dorsal ridges, two lateral ridges, and two ventral ridges. The posterior end of the cell is slightly extended into a small protrusion. Cell length is 26–30 μm, width is 22–27 μm. Anterior flagellum is about half of the cell length. The posterior flagellum is about 2.5 times longer than the cell and noticeably thicker than the anterior flagellum. Flagellar pocket is situated on the left side of the cell (viewed from the dorsal side) and the nucleus on the right side. Cells contain large food vacuoles in their posterior half. Well-marked ingestion organelle with two rods located on the right side, and expands towards the anterior end. Observed seven cells in LM.

Remarks: Morphology of observed cells corresponds with previous descriptions, except for the longer anterior flagellum

(equal to the cell length) and the shorter posterior flagellum (about 1.5 times longer than the cell length) (Ekebom et al., 1995; Larsen and Patterson 1990). This species is easily distinguished from other *Ploeotia* species by the almost round cells in outline and very prominent ridges.

Distribution: Marine waters of Australia (Ekebom et al., 1995), Fiji (Larsen and Patterson 1990), Red Sea (Tikhonenkov 2009). Fresh waters of European part of Russia (Tikhonenkov 2006; Tikhonenkov and Mazei 2007).

Ploeotia robusta Larsen and Patterson, 1990 (Fig. 5g–i)

Observations: Cells oval in outline, with oblique and acute posterior end (Fig. 5h). Cell length is 30–35 μm, cell width is 20–22 μm. Flagellar pocket opened subapically and covered laterally by a lip-like flange (Fig. 5h). The anterior flagellum is about the length of the cell. The posterior flagellum is 3.5 times longer than the cell and wider than anterior flagellum. One well-developed ventral groove extends from the lip-like structure to the posterior pointed tip (Fig. 5i), 4–5 slightly noticeable grooves are situated on both the ventral and dorsal sides of the cell (Fig. 5g). Well-marked ingestion organelle

with two gradually narrowing rods extends along the entire cell length. Nucleus located on the left side of the cell (viewed from the doorsal side). The posterior part of the cell contains a few food vacuoles. Cells lack any surface structure, except for the ventral groove associated with lip-like flanges, and a rough surface. Four cells were found in LM.

Remarks: We observed about 8–10 poorly visible grooves on the cell body surface, despite these not being part of the original description by Larsen and Patterson (1990). Patterson and Simpson (1996) also noticed at least eight delicate grooves arranged evenly around the cell body, which they described as being faintly visible and easily overlooked. At the same time, Al-Qassab with coauthors (Al-Qassab et al., 2002) recorded 3–4 ventral ridges and 4–5 dorsal ridges. Patterson and Simpson (1996) described longer flagella, with the anterior flagellum twice as long as the cell and posterior flagellum 4 times the length of the cell. This species differs from other *Ploeotia* species by larger cell size, presence of anterior-lateral lip-like flanges with extended well-developed ventral groove, long posterior flagellum, and posterior pointed end of the cell.

Distribution: Marine waters of U.K., Hawaii (Larsen and Patterson 1990). Hypersaline water of Australia (Al-Qassab et al. 2002; Patterson and Simpson 1996).

***Diplonemea Cavalier-Smith, 1993
****Diplonemidae Cavalier-Smith, 1993

***Diplonema ambulator* Larsen and Patterson, 1990 (Fig. 5j–m)

Observations: Cells are highly metabolic, can twist and contract to almost rounded in outline during turns (Fig. 5l). Cells are 14.5–16 μm long, 4–6 μm wide, elliptical with a slightly rounded posterior end, and an obliquely cut off anterior end. Equal-sized thin flagella emerge subapically from the prominent flagellar pocket, length of flagella is 5–6 μm. Flagella extend more or less perpendicular to axis of the cell. Cells mostly glide along the surface flexing their flagella, which gives an impression of "walking legs". J-shaped ingestion organelle seen during metaboly (Fig. 5l, m). The cell surface is bumpy. Only gliding cells were observed. Observations based on findings of six cells in LM.

Remarks: Morphology of studied cells mostly corresponds with previous descriptions, except that shorter flagella were described by Larsen and Patterson (1990), 3–4 μm; and longer flagella were described by Tong (1994, 1997b), 8 μm. Also, Tong (1994) noted that one flagellum sometimes can wraps around the cell, but we did not observe this. Other known species of this genus, *D. breviciliata* Griessmann, 1914, and *D. metabolicum* Larsen and Patterson, 1990, can be distinguished by cells being twice as large (Tong et al. 1998); *D. aggregatum* Tashyreva et al., 2018 and *D. japonicum* Tashyreva et al., 2018 can be distinguished by the broader anterior half of the cell that is slightly constricted and bent at the cell apex, as well as the presence of a sessile rounded cell stage and swimming stage with unequal flagella twice the length of the cell (Tashyreva et al. 2018); *D. papillatum* (Porter, 1973) Triemer and Ott, 1990 can be distinguished

by oval to the almost round outline of cells, the presence of prominent apical papilla, and very short, thick flagella (Porter 1973). Morphology of studied species is similar to *Rhynchopus amitus* Skuja, 1948 by its cell size and shape, but latter species can be distinguished by the presence of flagella that are thicker, barely emerge from the flagellar pocket, curve posteriorly, and are mostly motionless (Al-Qassab et al. 2002; Tong et al. 1998), see below.

Distribution: Marine waters of Australia (Tong et al. 1998), Brazil (Larsen and Patterson 1990), U.K. (Patterson et al. 1993; Tong 1994, 1997c).

***Rhynchopus amitus* Skuja, 1948 (Fig. 5n, o)

Synonym: *R. conscinodiscivorus* Schnepf, 1994.

Observations: Cells sac-shaped, with rounded ends and fine anterior apical papilla. Cell length is 9.5–12 μm, width is 4–5.5 μm. Cells very metabolic, periodically contracts. Flagellar pocket opened below the apical papilla, directed anterior-laterally. Flagella usually do not emerge from pocket. Numerous granules are visible inside the cell just below the cell surface. Cells glide slowly, constantly change directions; swimming cells were not observed. Two cells were found in LM.

Remarks: Most authors observed larger cells, up to 25 μm (Al-Qassab et al. 2002; Lee, 2015; Schnepf 1994; Skuja 1948; Tong et al. 1998). The species examined here differs from other species of *Rhynchopus* by cell shape and size. *R. euleeides* Roy et al., 2007 differs by the presence of long unequal flagella in swimming cells (2.5 times longer than the cell), symmetric elliptical shape of resting cells, clusters of many cells in culture (Roy et al. 2007). *R. hemris* Tashyreva et al., 2018 differs by both narrowed ends of the cell, swimming cells with one flagellum wrapped around anterior part of the cell and wobbling like a lasso, and another flagellum extended along the cell and waving (Tashyreva et al. 2018). *R. serpens* Tashyreva et al., 2018 has tapered anterior part of the cell and broad posterior end, as well as larger cell size (Tashyreva et al. 2018). Observed species is also similar to *Diplonema ambulator* (see above).

Distribution: Marine waters of Australia (Lee 2015; Lee et al. 2003; Tong et al. 1998), Wadden Sea ([as *R. conscinodiscivorus*] Schnepf, 1994). Saline inland waters of Romania ([as *Menoidium astasia*] Entz 1883). Hypersaline waters of Australia (Al-Qassab et al. 2002; [as *Menoidium astasia*] Ruinen 1938). Fresh waters of Sweden (Skuja 1948).

Incertae sedis EUKARYA: "CRuMs"

*"CRuMs" (Brown et al. 2018) [Varisulca Cavalier-Smith 2012]

***Mantamonas* Cavalier-Smith and Glücksman, 2011

***Mantamonas plastica* Glücksman and Cavalier-Smith in Glücksman et al., 2011 (Figure 6a–c)

Observations: Cells ovoid or triangular, highly metabolic, with posterior end extended into a changeable tail pseudopodium, connected with the posterior flagellum. The left side of the cell (viewed from the dorsal side) is smaller than the right side. Cell length is 3.5–4.5 μm, width is 2.5–3.0 μm at the broadest part. The anterior flagellum is thin, directed

laterally (to the left), and moves rarely. The length of anterior flagellum is equal to the length of the cell. The posterior flagellum is about twice the length of the cell, thicker than the anterior flagellum, and trails behind the cell. Both flagella non-acronematic. Ten cells were observed in LM.

Remarks: Size and morphology of observed cells are similar to those observed earlier, except in original description authors noted acronematic posterior flagellum (Glücksman et al. 2011). This species differs from other flagellates by the small size of its cells, the lateral orientation of anterior flagellum, and the flexible triangular shape of cells.

Distribution: Marine waters of U.K. (Glücksman et al. 2011), Australia (Lee 2015).

Other *incertae sedis* EUKARYA

"*Heterochromonas opaca*" *sensu* Lee and Patterson, 2000 (Fig. 6d, e)

Observations: Oval cells with slightly narrowed ends and apical anterior protrusion. Cell length is 11–15 μm, cell width is 8.5–11.0 μm. Flagella emerge from the ventral subapical depression at the base of the apical protrusion. The anterior flagellum is half the length of the cell, directed anterior-laterally, and mostly does not move. The posterior flagellum is 1.1–1.2 times the length of the cell, located laterally in a curve, and trailed behind the cell. Nucleus located anteriorly. The surface of the cells is warty. Cells move in a wide circle in a clockwise direction. Three cells were observed in LM.

Remarks: Morphology of cells we observed agreed well with cells described by Lee and Patterson (2000) as "*Heterochromonas opaca*". However, despite the similar cell shape, the cell originally described as *Heterochromonas opaca* Skuja (1948) is probably a different organism. The genus *Heterochromonas* Pascher, 1912 was introduced for colorless chrysomonads without scales, related to *Ochromonas* Vysotskii, 1887 and, probably, a junior synonym of *Spumella* Cienkowski, 1870 (Grossmann et al. 2016). Species of *Heterochromonas* are characterized by a clear stramenopile body plan, with non-flattened metabolic cells, truncated apically, and with two unequal heterodynamic flagella, both directed anteriorly, and with a small protoplasmic collar around apical depression. The species *Heterochromonas opaca* (Skuja 1948) is characterized by globular cells with a well-developed amoeboid collar that can almost completely disappear (except for the basal part), and the presence of short finger-like irregular pseudopodia involved in the capture of food particles. The cell described by Lee and Patterson (2000) as "*Heterochromonas opaca*" cannot be attributed to Chrysophyceae due to the rigid and flattened cells without an anterior collar and pseudopodia, and the long flagellum directed posteriorly and trailed behind the cell. *Heterochromonas opaca* (Skuja 1948) is a freshwater species with 2–3 contractile vacuoles, while "*Heterochromonas opaca*" *sensu* Lee and Patterson, 2000 was found only in marine waters (see distribution). Thus, most likely Lee and Patterson described a completely different species, probably new, that needs to be renamed and investigated to clarify its relationships with other eukaryotes.

According to the external morphology and movement, this organism could tentatively be attributed to Euglenida.

Previously described cells from the Black Sea (Prokina et al. 2018), identified as *Heterochromonas opaca* Skuja, 1948, are characterized by markedly different morphology: circular cells in outline; poorly developed anterior protrusion; smaller cell sizes; greater length and mobility of flagella. Probably it was another, undescribed species, related to "*Heterochromonas opaca*" *sensu* Lee and Patterson, 2000.

Distribution: Marine waters of Australia (Lee and Patterson 2000), South Korea (Lee 2002).

"Protist 1" (Fig. 6f–j)

Observations: Rigid cells are either spherical or ovoid, with a slightly flattened ventral side. Cell length is 4.5–5.5 μm, cell width is 3.8–5.5 μm. Unequal non-acronematic flagella insert subapically from the ventral side. The anterior flagellum is about the cell length, directed mostly forward, without moving, but can turn back when the cell turns (Fig. 6g, h). The posterior flagellum is about 25–35 μm long, trailed behind the cell. Pseudopodia were not observed. Nucleus located medially. Cells move slowly, gliding on the posterior flagellum. Four cells were observed in LM.

Remarks: Observed organisms differed from other known flagellate species by the presence of a very long posterior flagellum relative to the cell length. Long posterior flagellum and gliding cells are typical for *Glissandra innuerende* Patterson and Simpson, 1996 and *G. similis* Lee, 2006. The latter species differ by the following morphological features: longer anterior flagellum almost equal in length to posterior flagellum; flagella insert from subapical depression; cells glide on both flagella, and anterior flagellum always directed anteriorly; *G. similis* has a ventral longitudinal groove (Al-Qassab et al. 2002; Lee 2006). 'Protist 1' is also similar to *Sinistermonas sinistrorsus* Lee, 2015 which is distinguished from our taxon by its longer flagellum, directed anterior-laterally, and beating always to left in a small excursion (viewed from the dorsal side) (Lee 2015).

"Protist 2" (Fig. 6k–p)

Observations: Cells rigid, triangular in outline, laterally flattened, with tapered and curved ends, dorsally convex and ventrally concave with a large longitudinal groove that widens to one end of the cell. Cell length is 4.5–6.0 μm, width is 3.0–3.5 μm. Cells also slightly curved in the longitudinal axis (Fig. 6o). Flagella usually not seen, except possibly through perturbations of water at the edges of the cell. Sometimes a very thin flagellum (or filopodium?) is visible under the cell (Fig. 6m, n), which makes circular movements, potentially helping the cell move. Cells are located near a substrate and rotate around its axis. A stalk was not observed. Eleven cells were found in LM.

Remarks: Observed organism was frequently found in samples from the Black Sea and White Sea (unpublished). We can not exclude the possibility of investigating the dividing or dying cells of some known species. Very thin obscure flagellum also known for flagellate species *Ministeria vibrans* (Mylnikov et al. 2019). The presence of a
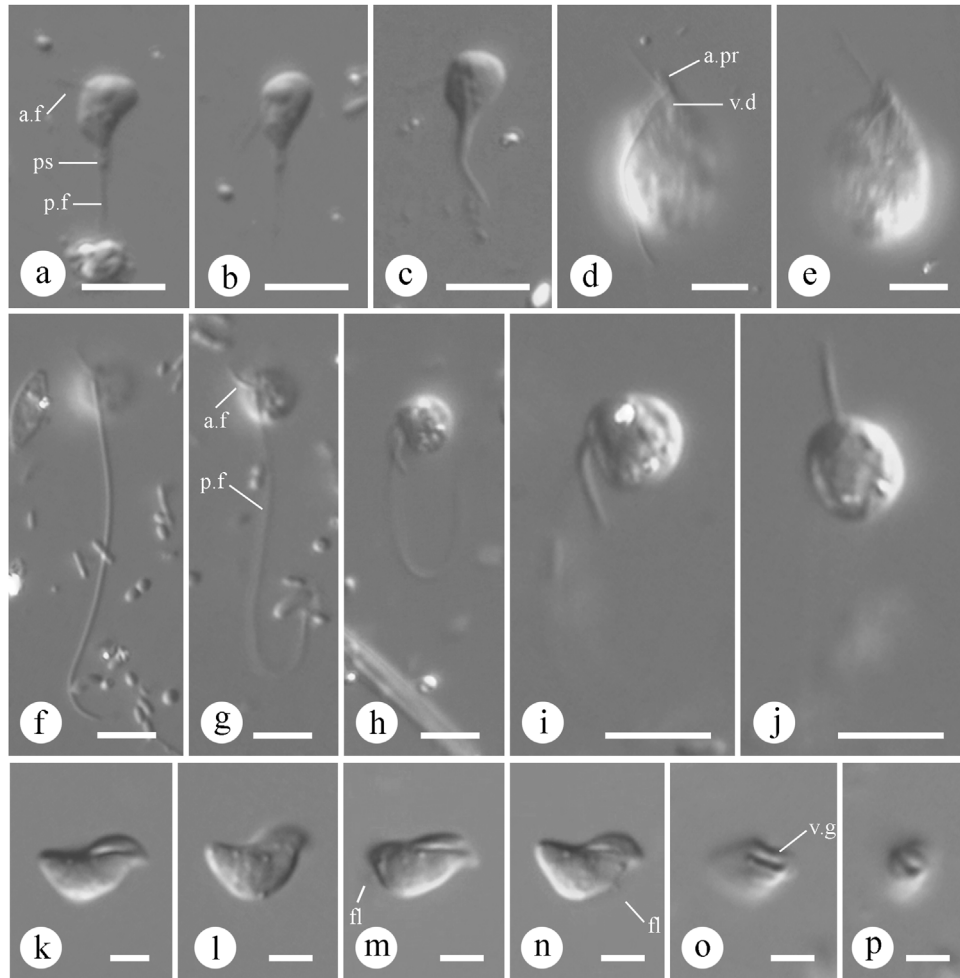
Fig. 6. Morphology of observed heterotrophic flagellates (DIC): a–c – *Mantamonas plastica*; d, e – "*Heterochromonas opaca*"; f–j – "Protist 1"; (f – both flagella in focus; g, h – cell turns; i, j – cell and anterior flagellum in focus); k–p – "Protist 2" (k–n – view from lateral side; o, p – view from ventral side, with ventral groove). Abbreviations: a.f – anterior flagellum; a.pr – apical protrusion; fl – flagellum; p.f – posterior flagellum; ps – pseudopodia; v.d – ventral depression; v.g – ventral groove. Scale bars: 5 μm.

longitudinal ventral groove is typical for Jakobida Cavalier-Smith, 1993. Among Jakobida there are some species in the family Histionina Cavalier-Smith, 2013, which also attach to the substrate and have a very similar cell shape and size: *Histiona aroides* Pasher, 1942, *H. velifera* (Voigt, 1901) Pasher, 1943, *Reclinomonas americana* Flavin and Nerad, 1993, and *R. campanulata* (Penard, 1921) Flavin and Nerad, 1993. These species, however, differ by their possession of lorica and clearly observable flagella. Aloricate Jakobids (such as *Ophirina amphinema* Yabuki et al., 2018, *Andalucia godoyi* Lara et al., 2006, *Moramonas marocensis* Strassert et al., 2016, etc.) are characterized by oval or bean-shaped cells with thick visible flagella, as well as typically swimming cells, not attached to the substrate (Lara et al. 2006; Strassert et al. 2016; Yabuki et al. 2018).

"Protist 3" (Fig. 7a–e)

Observations: Cell oval in outline, rigid, with a rounded anterior end, narrowed posterior end, and convex dorsal side. Not metabolic. Cell length is 7.5–10.0 μm, cell width is 5.5–6.5 μm. The entire surface of the cell is covered with prominent tubercles. Flagella thick, non-acronematic, and inserted apically from the anterior end of the cell. The anterior flagellum is 6.5–8.0 μm in length, directed anteriorly, slightly curved to the dorsal side (forming an arc), and moving very little. The posterior flagellum is 14–17 μm in length, trailed behind the cell. Flagellates slowly glide or swim. Swimming cells vibrate together with the flagella, and slowly rotates in different directions. The flagella keep the position like in gliding cells. Eight cells were examined in LM.

Remarks: Observed organism is most similar to glissomonads (*Allapsa*, *Sandona*, *Heteromita* etc.) and ancyromonads (*Ancyromonas*, *Planomonas*) in the shape and size of the cell. However, these species possess less rigid cells and a smooth cell surface without tubercles. Also, none of these species have a fixed arc position of flagella.

"Protist 4" (Fig. 7f–j)

Observations: The cell is 19.5–28.0 μm in length, 8–11 μm in width in the expanded part. Cells pear-shaped, highly
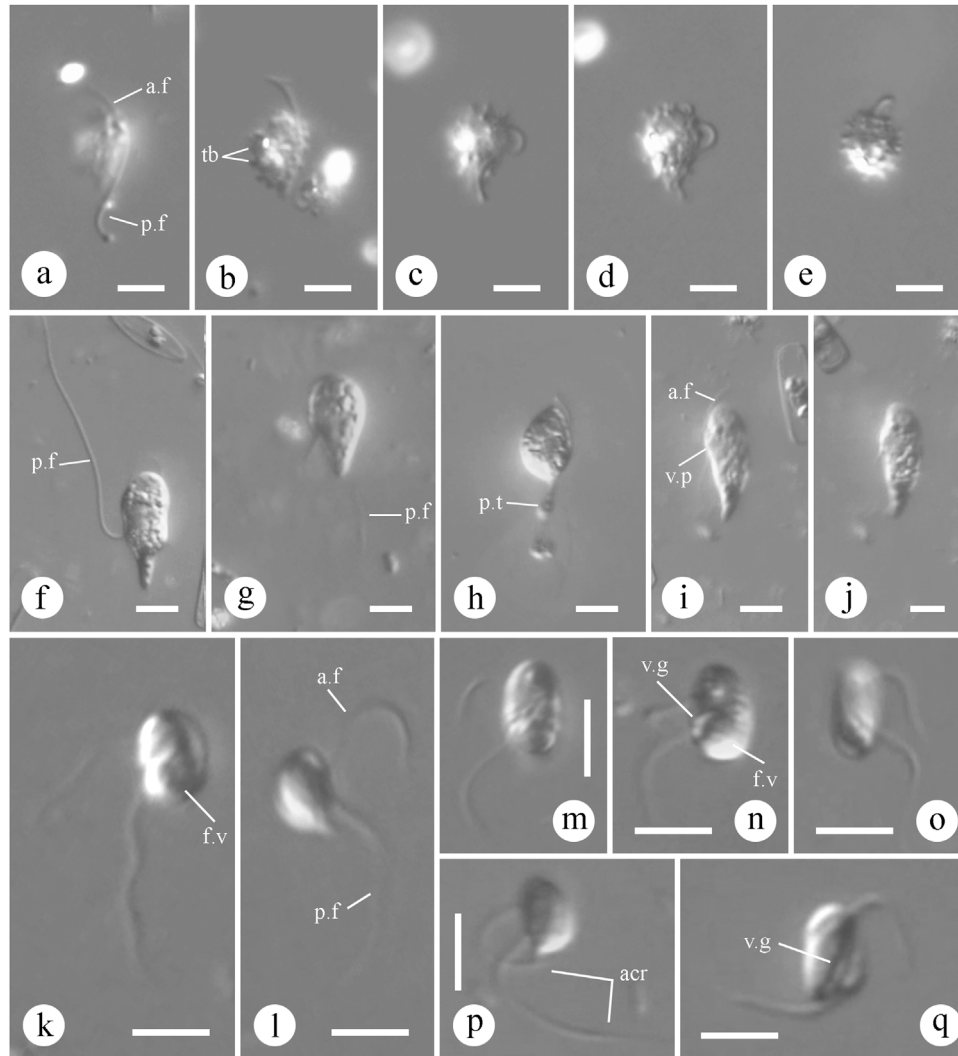
Fig. 7. Morphology of observed heterotrophic flagellates (DIC): a–e – "Protist 3"; f–j – "Protist 4" (f – non-swimming cell; g–j – swimming cells); k–q "Protist 5" (l, p – narrow starved cells; k, m, n – saturated cells with prominent food vacuoles; n–q – ventral groove (depression)). Abbreviations: acr – acronema; a.f – anterior flagellum; f.v – food vacuole; p.f – posterior flagellum; p.t – protoplasmic tail; tb – tubercles; v.g – ventral groove; v.p – ventral protrusion. Scale bar: 5 μm.

metabolic, with a posterior protoplasmic tail. Flagella insert subapically from ventral protrusion. Anterior flagellum thin, slightly noticeable during cell motility, directed forward and undulating. Posterior flagellum thicker vibrates with a constant amplitude of approximately 10 μm. Cells move slowly and swim in a straight line near the substrate, with a little trembling. Feeding behavior was not recorded, but probably food particles are captured by the caudal or ventral pseudopodia. Specimens were noted both in hypersaline and marine biotopes, with no difference in morphology. Six cells were examined in LM.

Remarks: Similar cell shape and trembling movement is observed in the flagellate genera *Aquavolon*, *Tremula*, and *Lapot*. However, these genera all share a clear diagnostic feature: the basal part of the posterior flagellum passes inside the cell and emerges at the central part of the body from the ventral opening. Species of the genus *Tremula* (*T. longi-*

fila Howe and Cavalier-Smith, 2011 and *T. vibrans* (Sandon, 1927) Cavalier-Smith, 2011) glide on the substrate (Howe et al. 2011), while 'Protist 4' was only observed swimming. Species of the genus *Aquavolon* (*A. dientrani* Tikhonenkov, Mylnikov and Bass, 2018, and *A. hoantrani* Tikhonenkov, Mylnikov and Bass, 2018) constantly rotate around the longitudinal axis of the cell during swimming (Bass et al. 2018), while 'Protist 4' swam straight. A similar manner of movement has been described for the species *Lapot gusevi* Tikhonenkov, Mylnikov, Irwin and Keeling, 2019 (Irwin et al. 2019), however, the latter is distinguished by a flattened cell and the presence of few short wide pseudopodia extending from the entire surface of the cell. In addition, known species of *Lapot* and *Aquavolon* were recorded only from freshwater.

"Protist 5" (Fig. 7k–q)

Observations: Small and very fast-moving oval cells, 3.5–7.0 μm in length, 2.5–4.5 μm in width, with two acrone-

matic flagella and anterior nucleus. Flagella insert from the ventral groove (depression) which begins just above the middle of the cell and goes obliquely to the posterior end of the cell. Posterior flagellum longer than the anterior, and both are longer than the cell. Flagella and their beating are almost invisible when the cell is moving. Cells swim rapidly and chaotically, always rotate in different planes, and change the direction of movement frequently. Sated cells are wide-oval with prominent posterior food vacuole (Fig. 7k, m, n). Starved cells are narrower, one side is more flattened and the other is more roundish, with the pointed posterior end (Fig. 7l, p). Eight cells were examined in LM.

Remarks: The presence of large posterior food vacuole may indicate a eukaryotrophic feeding mode of this protist. Fast-moving cells with ventral groove resemble colponemids, *Acavomonas,* and *Ancoracysta* (Janouškovec et al. 2017; Tikhonenkov et al. 2014). But the latter are twice the size and slower moving cells. Also, longitudinal grooves in colponemids and *Ancoracysta* do not go obliquely. Starved cells resemble some jakobids and *Cafeteria*-like stramenopiles in body plan, but their movement and behavior are absolutely different. Cells with planar ventral and convex dorsal surface are similar to bacteriovorous *Malawimonas* and some *Carpediemonas*-like organisms. But their flagella insert more anteriorly and are well defined. Also, *Malawimonas* swims in a straight line and known only from freshwater (O'Kelly and Nerad, 1999).

Discussion

The morphology of many of the observed species was slightly different from previous descriptions of any protist. Seven flagellate species were identified only to genus level: *Thecamonas* sp., *Colpodella* sp., *Cyranomonas* sp., *Goniomonas* sp., *Petalomonas* sp., *Ploeotia* sp. 1, and *Ploeotia* sp. 2. These organisms may represent not yet described species of these genera, but further studies are needed. Among the 86 heterotrophic flagellates and 3 centrohelids encountered in this survey (Table 2), five heterotrophic flagellates ("Protist 1", "Protist 2", "Protist 3", "Protist 4", and "Protist 5") and one centrohelid heliozoan (*Heterophrys*-like organism) were not identified even to the genus level. Of them, several of the flagellate protists have a unique morphology and may represent undescribed lineages of even a high taxonomic level. From the morphological perspective, we speculate that "Protist 1", "Protist 3", and "Protist 4" may be unknown lineages of marine cercozoans. "Protist 5" (resembling *Ancoracysta* and colponemids) and "Protist 2", which shows no significant similarity to known unicellular eukaryotes, may represent unknown (perhaps deep) branches of the tree of eukaryotes. Several such "weird-looking" protists have indeed recently been shown to represent new lineages (Janouškovec et al. 2017; Eglit, Simpson 2018; Eglit et al. 2017). The under-studied and puzzling *Hemimastix* similarly turned out to be a new, high-ranking phylogenetic

lineage of eukaryotes (Lax et al. 2018). Predatory protists with Colponemid-like morphology and behavior are also very intriguing and evolutionarily important, as they are falling in several different parts of the tree, and contribute to the understanding of the origin of photosynthesis, parasitism, and evolution of mitochondrial genome (Gawryluk et al. 2019; Janouškovec et al. 2013, 2017; Tikhonenkov et al., 2020b).

The greatest number of identified flagellate species seem to be members of the SAR supergroup (29 species), Excavates (27), and Obazoa (17). The lower number of flagellate species are apparently members of Cryptista (3), "CRuMs" (2), and Ancyromonadida (1). Six species are of an uncertain systematic position. Centrohelids fall into three families: Raphydocystidae (1 species), Choanocystidae (1), and Heterophryidae (1). All described protist species are new for marine waters of Curaçao.

More than half of the identified species of heterotrophic flagellates (46 species, 63%) and all identified centrohelid heliozoans were previously recorded not only from marine but also from freshwater habitats and can be considered as euryhaline. Twenty-seven flagellate species (37%) were previously reported only from marine and saline inland waters: *Acanthocorbis camarensis, Acanthoeca spectabilis, Bicosoeca maris, Caecitellus parvulus, Clautriaviabiflagellata, Colponema marisrubri, Cyranomonas australis, Diplonema ambulator, Discocelis punctata, Clissandrainnuerende, Goniomonas pacifica, Halocafeteria seosinensis, Mantamonas plastica, Massisteria marina, Metromonasgrandis, Paraphysomonas foraminifera, Percolomonas denhami, P. similis, Petalomonascantuscygni, Ploeotia punctata, P. robusta, Protaspategere, Salpingoeca infusionum, Stephanoeca cupula, S.diplocostata, S. supracostata, Volkanus costatus*.

The most frequent flagellate species in investigated sampling points were *Ancyromonas sigmoides, Cafeteria roenbergensis, Goniomonas truncata, Lentomonas azurina, Metopion fluens, Metromonas grandis, Neobodo designis, Petalomonas poosilla, Pseudophyllomitus apiculatus*, and *Rhynchomonas nasuta*; they were found in more than 20% of observed samples. Thirty flagellates were rare and found only in one sample. Each of the three observed centrohelid species was observed at only one sampling site.

The average number of species of flagellates and heliozoans in the studied samples is relatively low, 7.0 and 0.1 respectively. Studied marine biotopes have a significantly greater species diversity of flagellate species (total of 83 species, 7.2 on average), compared with hypersaline biotopes, (total of 17 species, 5.0 on average). Common for both marine and hypersaline waters were 15 flagellates: *Amastigomonas debruynei, Ancyromonas sigmoides, Bicosoeca maris, Caecitellusparvulus, Ciliophrys infusionum, Metopion fluens, Ministeria vibrans, Neobodo designis, N. saliens, Petalomonas poosilla, Ploeotia oblonga, Ploeotia* sp. 2, *Podomonas griebenis, Rhynchomonas nasuta*, and *Rhynchopus amitus. Halocafeteria seosinensis* and *Goniomonas* sp. were found only in hypersaline habitats.
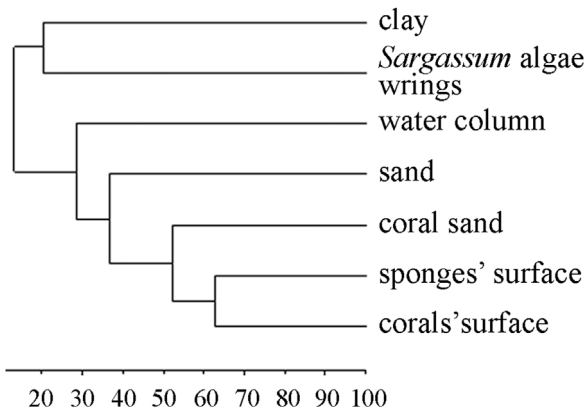
Fig. 8. Dendrogram showing the Dice similarity (%) of studied types of biotopes by species composition of heterotrophic flagellates.

Fig. 9. Species accumulation curve.

Among the observed types of biotopes, most flagellate species were found on the surface of corals (total of 57 species, on average – 8.1 species per sample) and the surface of sponges (39 and 7.2 respectively). These biotopes also were more similar in their species composition compared to other types of habitats (Fig. 8). But the largest number of species in the sample on average was found in coral sand (34 and 13.7). The lower total and the average number of flagellate species found in sand samples (24 and 3.9), water column (18 and 6.3), clay (8 and 4.5), and *Sargassum* algae wrings (2 and 2.0). Centrohelid heliozoans were observed only in scraping from a sponge (*Raphydocystis bruni*), sand (*Heterophrys*-like organism), and water column (*Choanocystis perpusilla*).

The vast majority (89%) of taxa identified to the species level are characterized by a generally wide geographical distribution and have previously been recorded from all hemispheres (North, South, West, and East), as well as from equatorial, tropical or subtropical, temperate, and polar regions. These (morpho)species can be considered cosmopolites (marked with "c" in Table 2). Seven species were previously described only from two regions: *Colponema marisrubri*, Red Sea (Tikhonenkov 2009) and Black Sea (Prokina et al. 2018); *Halocafeteria seosinensis*, Korea (Park et al. 2006) and South of European part of Russia (Plotnikov et al. 2011; Prokina 2020); *Petalomonas cantuscygni*, Brazil (Larsen and Patterson 1990) and U.K. (Cann and Pennick 1986); *Ploeotia punctata*, Australia (Al-Qassab et al. 2002; Larsen and Patterson 1990; Patterson and Simpson 1996) and Hawaii (Larsen and Patterson 1990); *Ploeotia robusta*, Australia (Al-Qassab et al. 2002; Patterson and Simpson 1996) and Hawaii (Larsen and Patterson 1990); *Mantamonas plastica*, U.K. (Glücksman et al. 2011) and Australia (Lee 2015); "*Heterochromonas opaca*", Australia (Lee and Patterson 2000) and Korea (Lee 2002). Among the listed rare species, about half (3) are relatively recently described (after 2000), and further surveys may expand data on their distribution. One species, *Clautriavia biflagellata*, was previously
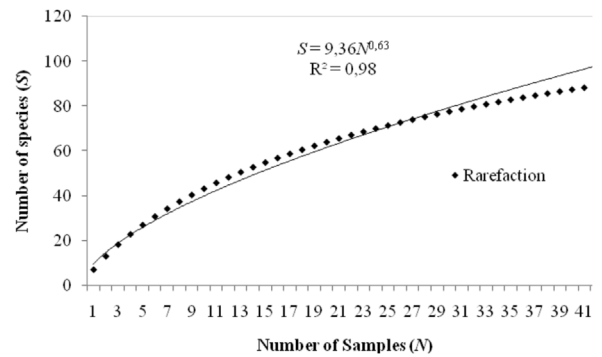
described only once, from Canada (Chantangsi and Leander 2010).

Among the identified heliozoans, *Choanocystis perpusilla* was recorded from all hemispheres and all the above-listed climatic regions. *Raphidocystis bruni* also has been observed in all hemispheres but only in temperate and tropical regions.

Nevertheless, the conclusions on the geographical distribution of protists are highly dependent on which species concept is applied (Azovsky et al. 2016). Here we followed the "morphospecies concept". However, analysis of the ribosomal RNA genes of several common flagellate morphospecies has shown that they can be represented by morphologically indistinguishable but genetically different strains (von der Heyden and Cavalier-Smith, 2005; Koch and Ekelund 2005; Pfandl et al. 2009; Scheckenbach et al. 2006; Scoble and Cavalier-Smith 2014a). Some of these strains appeared to be cosmopolitan, but others were not. In contrast, some benthic cercomonads, the stramenopile *Cantina*, the craspedid choanoflagellate *Codosiga,* and several acanthoecid species have all been shown to have no detected genetic divergence even between geographically distant populations (Bass et al. 2007; Nitsche and Arndt 2015; Stoupin et al. 2012; Yubuki et al. 2015).

Many regions of the world remain insufficiently studied for flagellates and even more so for heliozoans. Indeed, under-sampling is currently the key factor affecting our understanding of protists' diversity and distribution (Azovsky et al. 2020). To illustrate this, the species accumulation curve for studied Curacao samples (Fig. 9) fit well (R^2 = 0.98) with power function $S = 9.36N^{0.63}$. The curve does not flatten out (power coefficient is more than 0.5), so the species list obtained for these sites in Curacao is far from being complete, and each new sample should yield new species.

There is no doubt that there are many species unaccounted for in each protist diversity survey, and there are many unknown new species in natural ecosystems. Their discovery and description will not only clarify our understanding of distribution patterns of microeukaryotes but may also contribute to resolving previously puzzling evolutionary and ecological problems. Our observations from Curacao reveal many obscure species identified as "sp." or not identified at all. Future work on these protists and molecular

investigation in clonal cultures is very promising. Successful isolation in culture largely depends on the understanding of their biology and feeding mode, which can be clarified through observation in natural samples. Alternatively, single-cell transcriptomics is a very efficient approach to study molecular diversity, biology, and the functional state of even small-sized protists (Gavelis et al. 2015; Ku and Sebé-Pedrós 2019; Liu et al. 2017; Onsbring et al. 2019), which can be used for 'uncultivable' cells. Future discoveries and investigations of flagellates observed but unidentified here, and other unusual protists will complement the known diversity and biogeography of microbial eukaryotes and can also advance evolutionary research where these organisms represent new branches of the eukaryotic tree.

Author contributions

Acknowledgments

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