EXTENDED PHYLOGENY AND MORPHOLOGY OF MARINE AND FRESHWATER CHOANOFLAGELLATES WITH ADDITIONAL METHODOLOGICAL STUDIES ON HETEROTROPHIC FLAGELLATES

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„*Quam ob rem prius aequorum, amnium, stagnorumque dicentur.“*

„Daher soll zuvor [von den Organismen] in Meeren,
Flüssen und Seen die Rede sein.“

GAIUS PLINIUS SECUNDUS MAIOR, NATURALIS HISTORIA, IX, 1-2

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Zusammenfassung

Heterotrophe Flagellaten (HF) sind kleinzellige Protisten mit weltweiter Verbreitung. Als Hauptkonsumenten von Bakterien nehmen sie eine bedeutende Rolle im aquatischen und terrestrischen Nahrungsnetz ein. Um verlässliche Untersuchungen zur Diversität und Phylogenie der HF und besonders der Choanoflagellaten zu ermöglichen, wurde in der vorliegenden Arbeit ein einfach gehaltener Bestimmungsschlüssel für die gängigen, heterotrophen Flagellaten des Süßwassers entwickelt. Dieser Schlüssel soll die Bestimmung der diversen Morphotypen erleichtern, indem er auf morphologischen Charakteristika von Lebendorganismen basiert.

Aufgrund ihrer hohen Diversität und artspezifischer Eigenheiten ist es ferner schwierig, HF exakt zu quantifizieren. Daher wurden verschiedene Methoden zur Quantifizierung – die Lebendzählmethode, Fixierungsmethoden, Kultivierungs-methoden und Molekulare Arbeitsweisen – in Bezug auf Umweltstudien verglichen und jeweils optimiert. Die Vor- und Nachteile jeder dieser Quantifizierungsmethoden wurden gegenüber gestellt und Empfehlungen zur Wahl der Methoden in Hinblick auf die jeweilige zugrunde liegende Fragestellung gegeben.

Innerhalb der Vielfalt der Gruppen von HF wurde in der vorliegenden Doktorarbeit auf die Gruppe der Choanoflagellaten ein besonderes Augenmerk gerichtet. Als nächste einzellige Verwandte der Metazoa innerhalb der Gruppe der Opisthokonta ist diese Gruppe in evolutionärer Hinsicht - am Ursprung der Entwicklung zu tierischen Vertretern - äußerst interessant. Ihre systematische Einordnung, besonders diejenige der Ordnung Craspedida, wird noch immer kontrovers diskutiert, da bis heute keine monophyletischen Cluster bestätigt werden konnten. Zudem existieren in der Ordnung der Craspedida verschiedene morphologische Formen wie der Besitz oder das Fehlen einer Theka (Zellhülle aus organischem Material), aber auch verschiedene Ausprägungen des Lebenszyklus wie zum Beispiel die Fähigkeit, Kolonien zu bilden.

Zwölf Choanoflagellatenisolate aus weltweit verteilten marinen, Süßwasser- und Brackwasserhabitaten wurden mittels Sequenzierung ihrer ribosomalen 18S und 28S DNA untersucht, sowie ergänzend morphologisch beschrieben. Diese Arbeit konnte neue Erkenntnisse über die taxonomischen und systematischen Zusammenhänge der Vertreter der Ordnung Craspedida liefern. Fünf der isolierten Choanoflagellaten wurden zusätzlich in eine sechs Gene (18S rDNA, 28S rDNA, hsp90, tubA, EF-1A und EFL) umfassende phylogenetische Analyse einbezogen. Diese Analyse konnte zu einer optimierten phylogenetischen Auflösung und zu einem verbesserten Verständnis der Evolution der Choanoflagellaten beitragen.

Des Weiteren war es möglich, eine komplett neue Gruppe von bisher unbeschriebenen Choanoflagellatensequenzen aus suboxischen und anoxischen Habitaten zu charakterisieren. Diese neue Gruppe scheint nah mit der Choanoflagellatenordnung der Acanthoecida verwandt zu sein. Die Neubeschreibung dieser Gruppe wurde durch einen isolierten Choanoflagellaten ermöglicht, der eine neue Gattung begründete und erstaunlicherweise eine große morphlogische Ähnlichkeit zur Ordnung der Craspedida aufwies.

Zusammen genommen wurde durch die hier vorliegende Kombination von morphologischen und molekularen Daten die bestehende Sequenzdatenbank der Choanoflagellaten um ein Drittel erweitert, was eine mögliche Grundlage für eine umfassende Neuordnung der Choanoflagellaten und für Einblicke in ihre und die Evolution der Vielzelligkeit liefern könnte. Letztlich wurde außerdem erreicht, die schwierigen Prozesse der Choanoflagellatenisolierung, - kultivierung und –sequenzierung einen Schritt weiter zu optimieren. Zusätzlich wurden ökologische Untersuchungen zu Salztoleranz, Lorikabildung (Hülle der acanthoeciden Choanoflagellaten) und Fraßmechanismen durchgeführt.

Abstract

Heterotrophic flagellates (HF) are small, ubiquitously distributed protists. As main bacterial feeders, they play a significant role in aquatic and terrestrial food webs. To ensure reliable investigations of the diversity and phylogeny of HF and especially choanoflagellates, a short, user-friendly guide to common heterotrophic freshwater flagellates was developed in the present dissertation. It aims at facilitating an easier morphospecies identification using morphological characteristics of the living organisms.

Due to the large diversity and specific properties of HF, a reliable quantification of these organisms is very challenging. Different quantification methods such as the live-counting technique, various fixation methods, a cultivation method and molecular tools were thus compared and optimized regarding various environmental studies. On the basis of this comparison different recommendations have been provided.

Among the variety of different groups of HF, special attention was paid to the group of choanoflagellates in the present doctoral thesis. This group is particularly interesting regarding evolutionary aspects on the basis of animal origin as they are known to be the closest protistan relatives to the Metazoa within the group of Opisthokonta. Nonetheless, the internal systematics of choanoflagellates - especially of the order Craspedida - is still controversially discussed as no clear monophyletic clustering could be discovered up to now. Different morphological and life cycle forms exist, inter alia, the presence or absence of a theca (organic cell covering) and the ability to form colonies.

Here, the sequencing of the 18S and 28S ribosomal DNA and morphological description of twelve choanoflagellate isolates (marine, brackish, and freshwater) from world-wide sampling points could reveal new insights into the taxonomy and systematics of the order Craspedida.

Five of the isolates were additionally included in a six-gene phylogenetic analysis (18S rDNA, 28S rDNA, hsp90, tubA, EF-1A and EFL) to obtain an enhanced phylogenetic resolution and evolutionary understanding of choanoflagellates. Besides, it was also possible to characterize a completely new group of undescribed choanoflagellate sequences from suboxic/anoxic environments, closely related to the choanoflagellate order Acanthoecida. This group could be described by one isolate which was assigned to a new genus with a surprising morphological similarity to the order Craspedida.

Taken together, this combination of both morphological and molecular data extended the existing choanoflagellate sequence database by about one third and might provide the potential basis for a complete taxonomic revision of choanoflagellates and for insights into their evolution and the evolution of multicellularity. Furthermore, the difficult isolation, cultivation and sequencing processes of choanoflagellates could be partially optimized. Additional ecological studies were carried out regarding salinity tolerances, lorica inducement (covering of acanthoecid choanoflagellates) and feeding modes.

General Introduction and Aim of the Study

Heterotrophic flagellates (HF) are small and ubiquitously distributed protists. It is assumed that they are the most abundant eukaryotes on Earth (e.g. Lefranc et al. 2005); hundreds of specimens can be found in each droplet of water, even in groundwater and the deep sea. HF are the most important consumers of bacteria and are thus playing a key role in aquatic and terrestrial food webs (Arndt et al. 2000; Bonkowski 2004). The discovery of the microbial loop (Sorokin and Paveljeva 1972; Azam et al. 1983) with the cycling and transfer of biomass and energy to higher trophic levels highlighted this outstanding key role of HF.

Altogether, HF are a very heterogenous group of protists with cell sizes ranging from 1 to 450 µm. Different dietary preferences can occur: Herbivorous (Arndt and Mathes 1991; Nauwerck 1963; Sherr and Sherr 1994), detritivorous (Scherwass et al. 2005), osmotrophic (Christoffersen et al. 1997; Sanders et al.1989; Sherr 1988) and mixotrophic types (Bird and Kalff 1986; Sanders 1991). These diverse preferences are not related to taxonomy and vary even within one species, e.g. the mixotrophic *Ochromonas* sp. (Jones 2000; Wilken et al. 2013). HF can be true filter-feeders (e.g. choanoflagellates), direct interception feeders (e.g. chrysomonads) or raptorial feeders (e.g. most benthic forms) (Boenigk and Arndt 2002; Fenchel 1991). Various modes of movement can also be found amongst HF: gliding as well as free-swimming forms or forms, which are temporarily or permanently attached to substrate and again sometimes switching between different modes (Fenchel 1987). As a lot of HF can tolerate high changes in salinity, several species are living in both, marine and freshwater habitats, although a number of phylogenetic studies revealed clearly separated marine and freshwater clades (e.g. Kim and Archibald 2013). Despite all, the main taxonomic groups within different marine and freshwater pelagic (e.g. stramenopile taxa, dinoflagellates, choanoflagellates, kathablepharids) and benthic communities (e.g. euglenids, free-living kinetoplastids, cercozoans) appear to be – in general - strikingly similar (Arndt et al. 2000).

To ensure reliable studies on the different groups of HF with special emphasis on one group and to reveal their outstanding diversity, a short, user-friendly guide to common heterotrophic freshwater flagellates was developed in the present dissertation. The development of such a short but broadly covering guide – as developed by Foissner and Berger (1996) for ciliates – was still lacking for HF.

Among the variety of different groups of HF, one group is most peculiar regarding ecological and especially evolutionary studies. This is the group of choanoflagellates (Choanomonada). Species belonging to this group are often very difficult to isolate and cultivate. Yet, special attention was given to this group in the present dissertation. Choanoflagellates are small phagotrophic protists ubiquitously distributed in all aquatic habitats and even found in dry soil. In respect to the ecological importance, choanoflagellates possess a single apical flagellum surrounded by a collar of microvilli (Adl et al. 2012) which is used for filter-feeding on large amounts of bacteria (Arndt et al. 2000; Boenigk and Arndt 2002). Due to this highly efficient filter feeding, together with their high abundances in many different aquatic environments, they can have a huge impact on the microbial food web - despite their small cell size (for examples in different aquatic environments see e.g. for marine: Leadbeater 1974; Stock et al. 2012; Thomsen et al. 1997; for brackish: Wylezich and Jürgens 2011).

Regarding the evolutionary aspect, choanoflagellates are known to be the closest living relatives to Metazoa/animals within the group of Opisthokonta and are thus the ideal reference taxon to study the evolution of multicellularity (e.g. King et al. 2008; Richter and King 2013, see Fig. 1). In choanoflagellates, not only single celled phases, but also simple, multicellular colonies might be formed within their life history (Dayel et al. 2011). Phylogenetic and morphological studies of choanoflagellates might help reconstructing the origin of multicellularity, the cell biology and genome composition of the first animals. However, just about 37 reference sequences had been available before the beginning of the present work and the taxonomy and phylogeny of choanoflagellates is still controversially discussed.

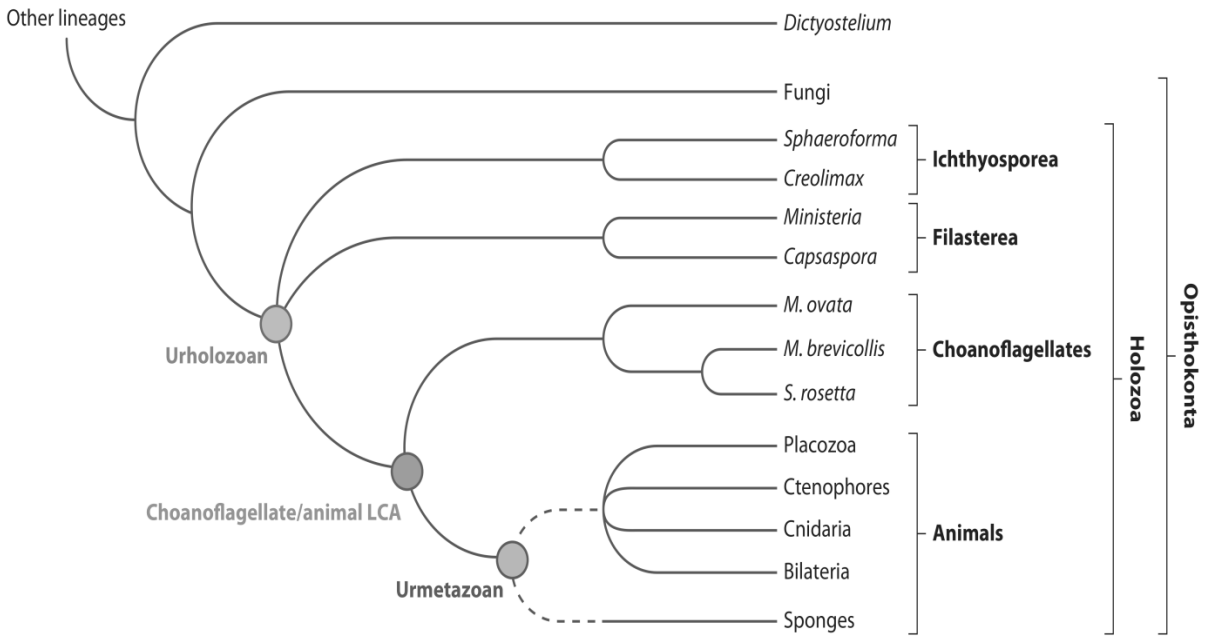

Figure 1. Phylogenetic framework for the reconstruction of the origin of multicellularity/animals. It might be possible to gain insights into the last common ancestor (LCA) of choanoflagellates and animals comparing both groups of organisms (taken from **Richter DJ, King N** (2013) The genomic and cellular foundations of animal origins. Annu Rev Genet **47:** 509–537).

Currently, choanoflagellates are classified into two orders according to the presence or absence of a lorica (silicified costae around the cell body) – Acanthoecida (loricates) and Craspedida (non-loricates, but sometimes possessing a theca, an organic cell covering). Molecular data, mainly based on SSU rDNA, show that on the one hand the phylogeny of loricate species is well defined and monophyletic families exist. On the other hand the two craspedid families of Salpingoecidae and Codosigidae, based on morphological characters, were abandoned as they were clearly not monophyletic (Nitsche et al. 2011).

Unfortunately, most choanoflagellate species were only described morphologically since their discovery (e.g. Saville Kent 1880-82). Due to the fact that cryptic diversity within morphospecies and life cycle forms exist (Stoupin et al. 2012; Dayel et al.

2011), a revised craspedid taxonomy with the help of morphological and especially molecular data is necessary.

Besides, the accurate quantification of environmental choanoflagellates and HF in general is very difficult as the development of reliable quantification methods has somewhat fallen into oblivion since the discovery of their importance for the microbial loop (e.g. Azam et al. 1983). Some methodological studies dealt with different HF quantification techniques in the last decades (Alongi 1991; Gifford and Caron 2000; Massana and Güde 1991; Sherr and Sherr 1993). But most of the quantitative data were restricted to heterotrophic nanoflagellates ("HNF") per se with a size range of ≤15µm. Little attention has been paid to the high taxonomic and spatial variability (Arndt et al. 2000). Most of these studies emphasized the use of fixed and stained samples, using epifluorescence microscopy for the cell counting (e.g. Caron 1983; Gifford and Caron 2000). However, fixation can bear biases. The live-counting (e.g. Gasol 1993; Massana and Güde 1991) and cultivation method – the liquid aliquot method (LAM, Butler and Rogerson 1995) or the most probable numbers (MPN, Sinclair and Ghiorse 1987) are other important alternative quantification techniques which should be taken into account. Furthermore, molecular tools have also been increasingly applied (Egge et al. 2013; Heywood et al. 2010; Lynn and Pinheiro 2009). Up to now, a detailed and broad study comparing long-term established non-molecular and newly developed molecular methods was still lacking. Yet, to ensure an accurate, reliable and optimized quantification of HF, a methodological comparison is long needed; this challenge was faced in the doctoral thesis.

To summarize, one aim of this dissertation was to establish a suitable identification tool for HF. This developed guide is intended to reveal the often underestimated huge diversity of HF and especially choanoflagellates in freshwater habitats. Within this HF diversity it was then focused on the particular choanoflagellates with special emphasis on their phylogeny and morphology: Several new species have been described, a new group of acanthoecid related choanoflagellates was discovered and a six-gene phylogeny has provided new insights into the craspedid phylogeny.

Working with HF in general and choanoflagellates in particular, has shed light on the fact that a comparison of different quantification methods is highly in demand. Hence, the present work has been accomplished by a methodological consideration to optimize HF quantification methods.

The present dissertation was subdivided into the following chapters. The order and numbering of the chapters 1 - 5 result from the guidelines for a cumulative dissertation.

In **chapter 1** a short guide to common heterotrophic freshwater flagellates was developed. The guide is designed as a user-friendly guideline for biologists not specialised in the study of HF in pelagic and benthic freshwater habitats. The aim was to give a reliable tool to unravel the black box of “HNF” (heterotrophic nanoflagellates) as most studies were restricted to HNF and disregarded the striking diversity of this protistan group. The guide is polytomously organised with the help of drawings and video sequences. Identification is based on the morphology of living organisms and a huge number of species morphotypes can be determined at least to genus level. Special attention was given to the most abundant and ecologically relevant forms. The guide is intended to be used for the live-counting quantification method - inter alia discussed in the last chapter of this thesis.

In **chapter 2** the phylogeny of the choanoflagellate order Craspedida was extended by adding six newly isolated species to the existing knowledge. These six species were isolated from saline, brackish, and freshwater habitats from world-wide sampling points. Two of the six species were assigned to previously described species. The sequencing of their 18S and 28S rDNA revealed new insights into the phylogeny and systematics of the Craspedida. Additionally, the question was raised, whether morphology (form of the theca) or habitat preference are reflected in the phylogenetic clustering of the Craspedida.

In **chapter 3** it was concentrated on one particular choanoflagellate isolate from the River Rhine, Cologne, Germany. Morphological studies characterised this species as belonging to the order Craspedida due to its typical form of craspedid choanoflagellates *(Monosiga*-like morphology). In contrast to that, the phylogenetic analysis (18S + 28S rDNA) of this species revealed a surprising relationship to the Acanthoecida, the other order of choanoflagellates. Thus, this isolate defined a new genus and species (*Acanthafallax monosigata)* within a clade of uncultured sequences mostly isolated from suboxic/anoxic freshwater, sediment and soil environments. Acanthoecid choanoflagellates are mostly described from marine habitats but *Acanthafallax monosigata* was isolated from freshwater. Thus, autecological experiments were performed to test the salinity tolerance of the newly described isolate to get hints regarding the origin of *Acanthafallax monosigata*. In addition, the ability of *Acanthafallax monosigata* to form a lorica was tested to gain insights into the origin and development of this special choanoflagellate species.

In **chapter 4** a phylogenetic dataset of 42 choanoflagellates was presented comprising six genes: 18S rDNA, 28S rDNA, hsp90, tubA, EF-1A and EFL. Several craspedid species and genera were taxonomically reordered and further five were newly isolated and described and new insights into the morphological and ecological evolution of the choanoflagellates were provided.

In **chapter 5** the problematic quantification of HF in environmental samples was faced. Thus, different quantification methods were compared elucidating the underlying advantages and disadvantages of the different methods (Fixation, live-counting, cultivation and molecular methods). Hence, a methodological survey of HF quantification techniques and recommendations of reliable methods regarding different problems were provided. Most of the environmental studies – used to demonstrate the different methods - were concentrated on the River Rhine in Germany as an important HF freshwater habitat.

Chapter 1

A short guide to common heterotrophic flagellates of freshwater habitats based on the morphology of living organisms

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PROTIST TOOLS

A Short Guide to Common Heterotrophic Flagellates of Freshwater Habitats Based on the Morphology of Living Organisms

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Introduction

Heterotrophic flagellates (HF) are very likely the most abundant eukaryotes on Earth, hundreds of specimens occur in each droplet of water even in groundwater and the deep sea. As the main feeders on bacteria they play an essential role in aquatic and terrestrial food webs (Arndt et al. 2000; Azam et al. 1983; Bonkowski 2004). In addition, they can act as important herbivores (Arndt and Mathes 1991; Nauwerck 1963; Sherr and Sherr 1994), detritivores (Scherwass et al. 2005) and osmotrophs (Christoffersen et al. 1997; Sanders et al. 1989; Sherr 1988) as well as mixotrophs (Bird and Kalff 1986; Sanders 1991). The relative contributions to these different modes of feeding can vary within taxonomic groups and even within one and the same organism, e.g. *Ochromonas* sp. (Jones 2000; Wilken et al. 2013). Furthermore, bacterial communities are not only grazed by protozoans but are also structured by protistan grazers (e.g. Boehme et al. 2009; Güde 1979; Jürgens and Matz 2002; Pernthaler 2005).

HF are a very heterogenous group with an enormous size range between 1-450 μm (some authors refer to the species smaller than 15 μm as heterotrophic nanoflagellates "HNF", Arndt et al. 2000). High tolerances to changes in salinity allow several species to live both in marine as well as in freshwater habitats, though several phylogenetic studies have also indicated clearly separated marine and freshwater clades (e.g. Kim and Archibald 2013). In contrast, the dominant taxonomic groups within different marine and freshwater pelagic communities (e.g. stramenopile taxa, dinoflagellates, choanoflagellates, kathablepharids) and benthic communities (e.g. euglenids, free-living kinetoplastids, cercozoans) seem to be surprisingly similar (Arndt et al. 2000). Their modes of movement as important taxonomic characteristics comprise gliding or free-swimming forms or they may be temporarily or permanently attached to a substrate (Fenchel 1987). Feeding modes include true filter-feeding (e.g. choanoflagellates), direct interception feeding (e.g. chrysomonads) or raptorial feeding (e.g. most benthic forms) (Boenigk and Arndt 2002; Fenchel 1991).

One important prerequisite to estimate the role of HF for the flux of matter in ecosystems is the determination of their abundance. In the early days of HF quantification, the so called "HNF" were counted exclusively in fluorescently stained fixated samples (e.g. Porter and Feig 1980). However, fixation bears the problem that many species are disrupted upon the fixation process (Choi and Stoecker 1989; Sonntag et al. 2000) or are misidentified as small naked amoebae, zoospores, yeasts, or disrupted cells from a range of eukaryotes, since flagella are not always adequately preserved (Patterson and Larsen 1991). Especially benthic HF are affected by the above explained problems because they are additionally masked by sediment particles. Further, large HF can easily be overlooked on membrane filters due to their low contribution to total HF abundance and their special sensitivity to fixatives. This is crucial since they may contribute to about half of the HF biovolume (Arndt et al. 2000). Therefore, the

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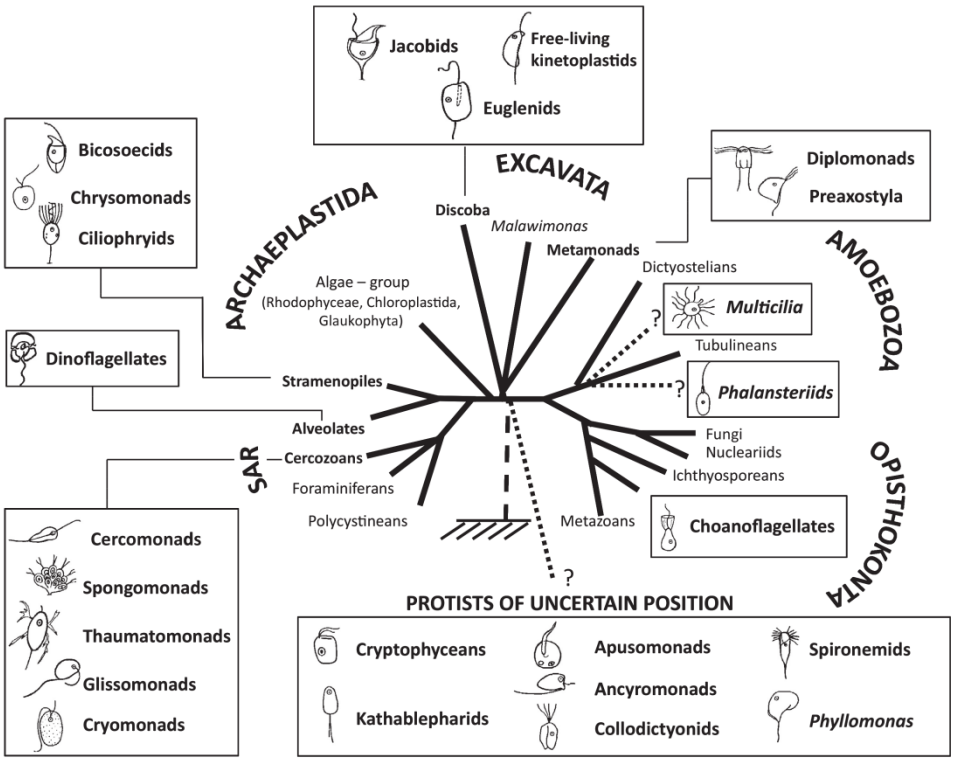

Figure 1. Selective overview of the systematic position (according to Adl et al. (2012) of flagellate groups considered in the guide (see Charts 1–15).

taxonomic resolution of fixation processes is often very low, most quantitative studies even ignored the variety of HF.

In contrast, live counting is an essential and reliable method for determining HF abundances. It allows a combination of taxonomic and quantitative studies for understanding the role of HF in microbial food webs (Leadbeater and Green 2000; Patterson and Larsen 1991). Unfortunately, the taxonomic resolution of HF studies is hampered by the fact that the literature is often focused on only a few taxonomic groups. Therefore, we designed a (hopefully) user-friendly guide for ecologists to unravel the "HNF" black box. In contrast to the few already existing taxonomic keys/reviews (e.g. Bass et al. 2009; Cavalier-Smith and Chao 2010; Lee et al. 2000; Patterson and Hedley 1992; Patterson and Larsen 1991; Zhukov 1993), the present guide is compact and focuses on the dominant and common forms of HF occurring in pelagic and benthic freshwater habitats. Following the idea of Foissner and Berger (1996) with regard to ciliates, we designed a guide which should be useful also for biologists not specialized in the study of heterotrophic freshwater flagellates due to its polytomous organization, the help of drawings and video sequences (Supplementary Material). According to our experience in quantitative studies of morphotypes, about 80-90 per cent of the dominant heterotrophic flagellates found in freshwater habitats should be identifiable at least to rough taxonomic categories (and functional guilds). The term morphotype is used here for taxa that can be differentiated by means of their morphology when only analysed by light microscopy. It means that e.g. genotypes may be hidden among certain morphotypes. A typical example is the species complex of *Neobodo designis* (Scheckenbach et al. 2006). The guide comprises all the main groups, so that a large number of species morphotypes can be determined at least to the level of genus. Special attention was given to the most interesting and ecologically relevant forms. The taxonomic categories were used according to the recently revised classifications by Adl et al. (2012).

Within the next decade, next generation sequencing of field samples will give more detailed insights into the structure of HF field communities

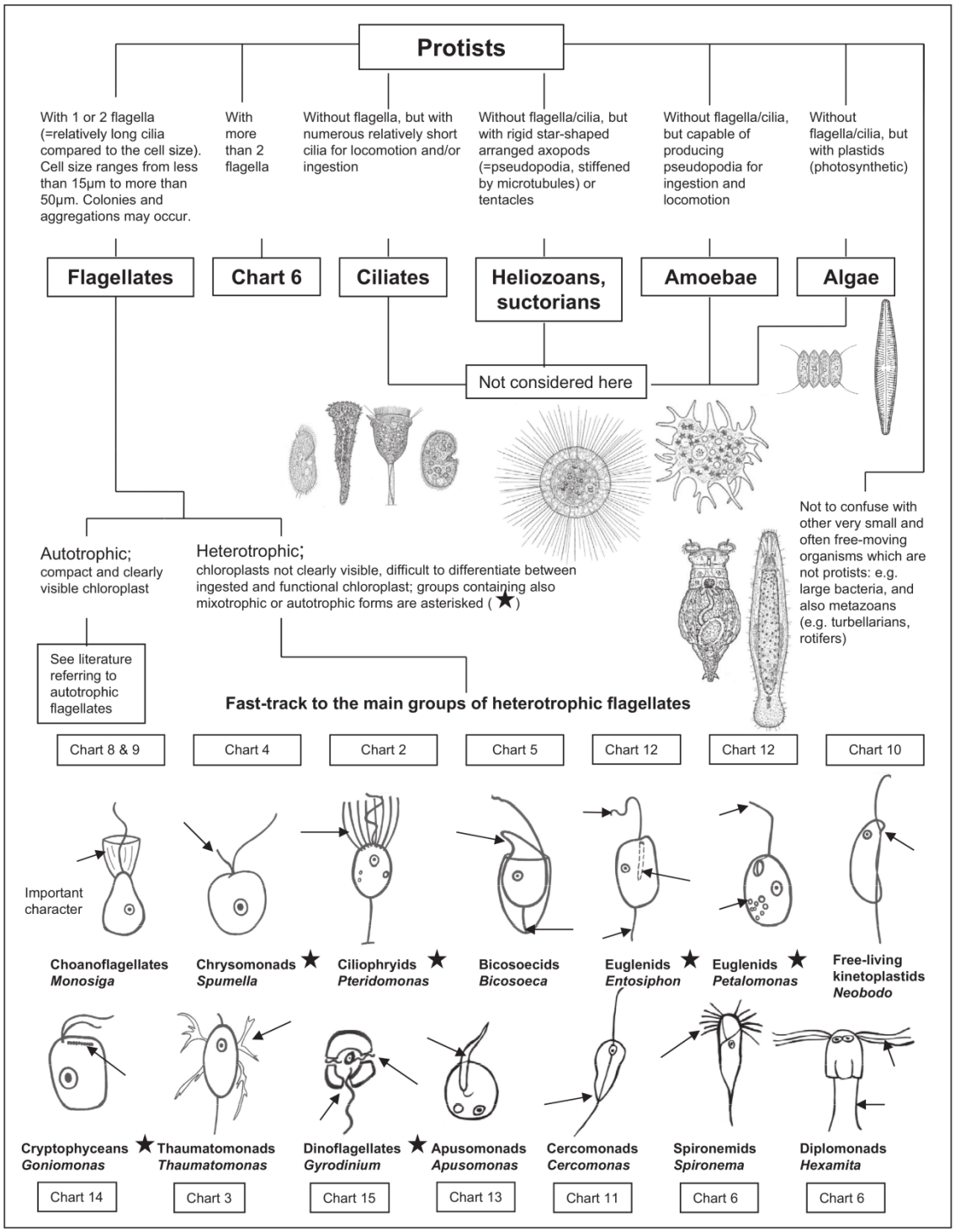

Chart 1. User-friendly guide to common heterotrophic freshwater flagellates. All scale bars in the guide indicate 10 µm.

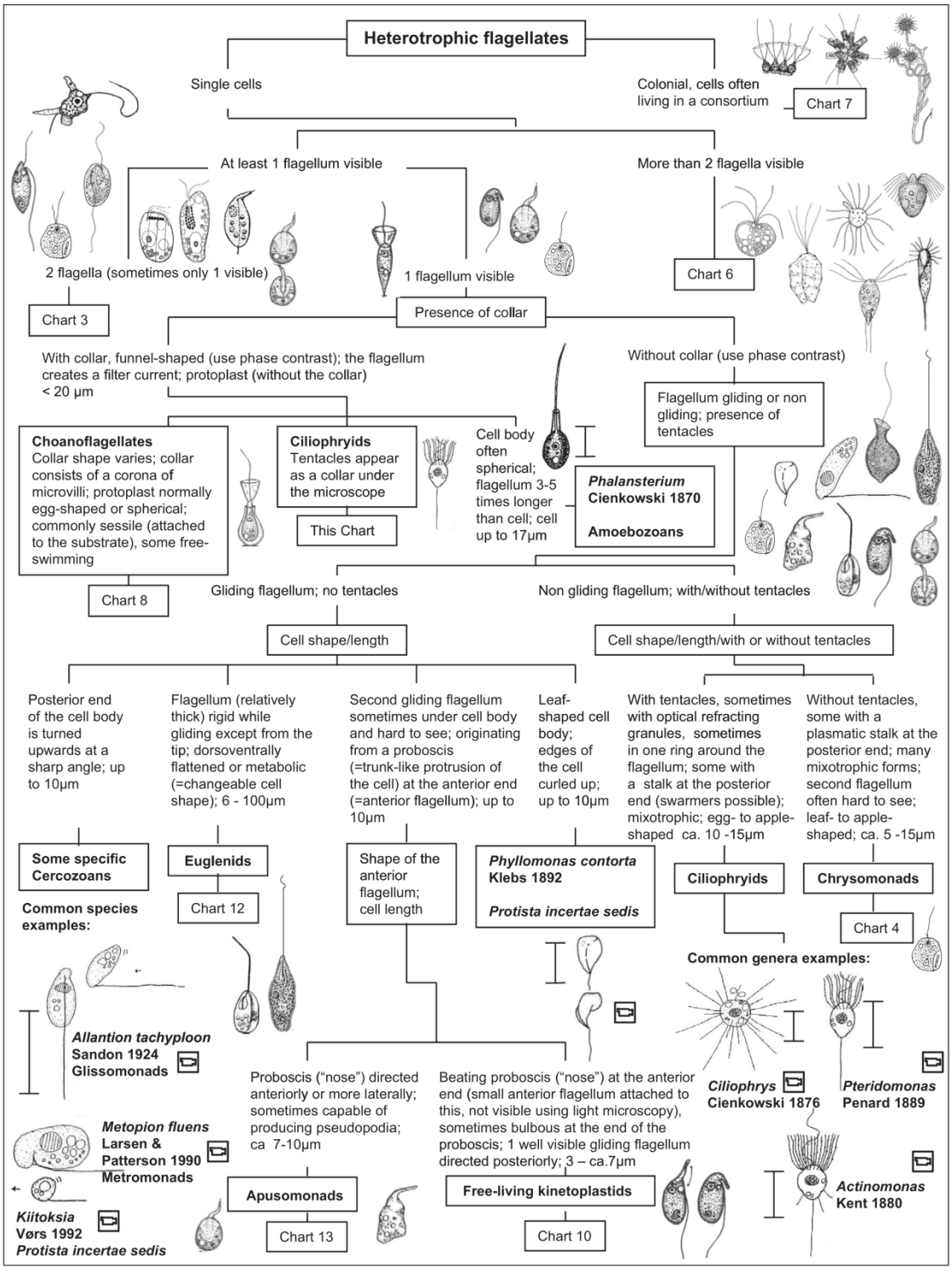
Heterotrophic flagellates
Single cells
Colonial, cells often living in a consortium
Chart 7
At least 1 flagellum visible
More than 2 flagella visible
2 flagella (sometimes only 1 visible)
1 flagellum visible
Chart 6
Chart 3
Presence of collar
With collar, funnel-shaped (use phase contrast); the flagellum creates a filter current; protoplast (without the collar) < 20 µm
Without collar (use phase contrast)
Flagellum gliding or non gliding; presence of tentacles
Choanoflagellates
Collar shape varies; collar consists of a corona of microvilli; protoplast normally egg-shaped or spherical; commonly sessile (attached to the substrate), some free-swimming
Ciliophryids
Tentacles appear as a collar under the microscope
This Chart
Cell body often spherical; flagellum 3-5 times longer than cell; cell up to 17µm
Phalansterium Cienkowski 1870
Amoebozoans
Chart 8
Gliding flagellum; no tentacles
Non gliding flagellum; with/without tentacles
Cell shape/length
Cell shape/length/with or without tentacles
Posterior end of the cell body is turned upwards at a sharp angle; up to 10µm
Flagellum (relatively thick) rigid while gliding except from the tip; dorsoventrally flattened or metabolic (=changeable cell shape); 6 - 100µm
Second gliding flagellum sometimes under cell body and hard to see; originating from a proboscis (=trunk-like protrusion of the cell) at the anterior end (=anterior flagellum); up to 10µm
Leaf-shaped cell body; edges of the cell curled up; up to 10µm
With tentacles, sometimes with optical refracting granules, sometimes in one ring around the flagellum; some with a stalk at the posterior end (swarmers possible); mixotrophic; egg- to apple-shaped ca. 10 -15µm
Without tentacles, some with a plasmatic stalk at the posterior end; many mixotrophic forms; second flagellum often hard to see; leaf- to apple-shaped; ca. 5 -15µm
Some specific Cercozoans
Euglenids
Shape of the anterior flagellum; cell length
Phyllomonas contorta Klebs 1892
Protista incertae sedis
Ciliophryids
Chrysomonads
Common species examples:
Chart 12
Chart 4
Common genera examples:
Allantion tachyploon Sandon 1924 Glissomonads
Ciliophrys Cienkowski 1876
Pteridomonas Penard 1889
Metopion fluens Larsen & Patterson 1990 Metromonads
Proboscis ("nose") directed anteriorly or more laterally; sometimes capable of producing pseudopodia; ca 7-10µm
Beating proboscis ("nose") at the anterior end (small anterior flagellum attached to this, not visible using light microscopy), sometimes bulbous at the end of the proboscis; 1 well visible gliding flagellum directed posteriorly; 3 – ca.7µm
Actinomonas Kent 1880
Kiitoksia Vørs 1992 Protista incertae sedis
Apusomonads
Free-living kinetoplastids
Chart 13
Chart 10

Chart 2.

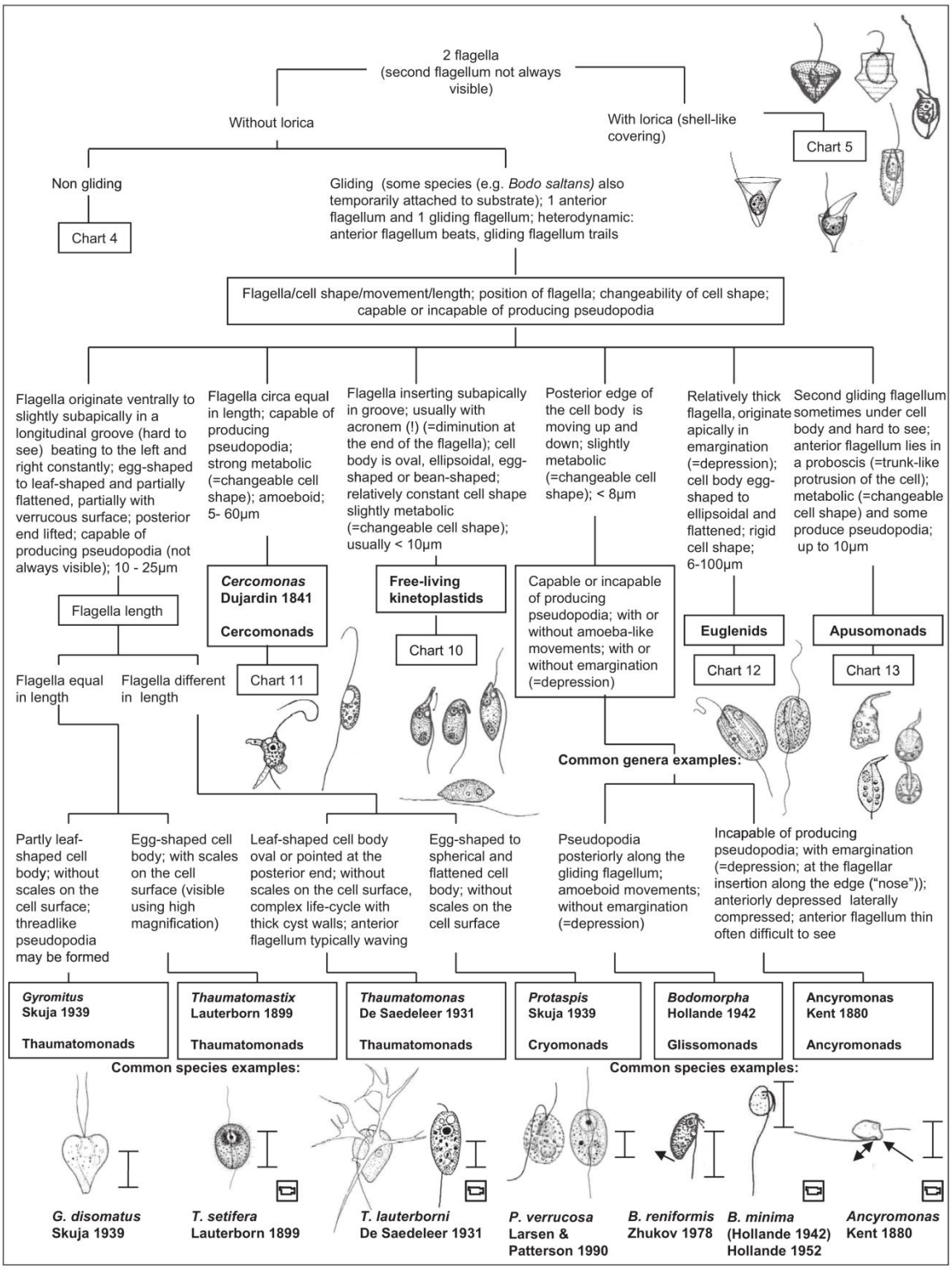
2 flagella
(second flagellum not always visible)
Without lorica
With lorica (shell-like covering)
Chart 5
Non gliding
Chart 4
Gliding (some species (e.g. Bodo saltans) also temporarily attached to substrate); 1 anterior flagellum and 1 gliding flagellum; heterodynamic: anterior flagellum beats, gliding flagellum trails
Flagella/cell shape/movement/length; position of flagella; changeability of cell shape; capable or incapable of producing pseudopodia
Flagella originate ventrally to slightly subapically in a longitudinal groove (hard to see) beating to the left and right constantly; egg-shaped to leaf-shaped and partially flattened, partially with verrucous surface; posterior end lifted; capable of producing pseudopodia (not always visible); 10 - 25µm
Flagella length
Flagella equal in length
Flagella different in length
Flagella circa equal in length; capable of producing pseudopodia; strong metabolic (=changeable cell shape); amoeboid; 5- 60µm
Cercomonas Dujardin 1841
Cercomonads
Chart 11
Flagella inserting subapically in groove; usually with acronem (!) (=diminution at the end of the flagella); cell body is oval, ellipsoidal, egg-shaped or bean-shaped; relatively constant cell shape slightly metabolic (=changeable cell shape); usually < 10µm
Free-living kinetoplastids
Chart 10
Posterior edge of the cell body is moving up and down; slightly metabolic (=changeable cell shape); < 8µm
Capable or incapable of producing pseudopodia; with or without amoeba-like movements; with or without emargination (=depression)
Common genera examples:
Relatively thick flagella, originate apically in emargination (=depression); cell body egg-shaped to ellipsoidal and flattened; rigid cell shape; 6-100µm
Euglenids
Chart 12
Second gliding flagellum sometimes under cell body and hard to see; anterior flagellum lies in a proboscis (=trunk-like protrusion of the cell); metabolic (=changeable cell shape) and some produce pseudopodia; up to 10µm
Apusomonads
Chart 13
Partly leaf-shaped cell body; without scales on the cell surface; threadlike pseudopodia may be formed
Egg-shaped cell body; with scales on the cell surface (visible using high magnification)
Leaf-shaped cell body oval or pointed at the posterior end; without scales on the cell surface, complex life-cycle with thick cyst walls; anterior flagellum typically waving
Egg-shaped to spherical and flattened cell body; without scales on the cell surface
Pseudopodia posteriorly along the gliding flagellum; amoeboid movements; without emargination (=depression)
Incapable of producing pseudopodia; with emargination (=depression; at the flagellar insertion along the edge ("nose")); anteriorly depressed laterally compressed; anterior flagellum thin often difficult to see
Gyromitus Skuja 1939
Thaumatomonads
Thaumatomastix Lauterborn 1899
Thaumatomonads
Thaumatomonas De Saedeleer 1931
Thaumatomonads
Protaspis Skuja 1939
Cryomonads
Bodomorpha Hollande 1942
Glissomonads
Ancyromonas Kent 1880
Ancyromonads
Common species examples:
Common species examples:
G. disomatus Skuja 1939
T. setifera Lauterborn 1899
T. lauterborni De Saedeleer 1931
P. verrucosa Larsen & Patterson 1990
B. reniformis Zhukov 1978
B. minima (Hollande 1942) Hollande 1952
Ancyromonas Kent 1880

Chart 3.

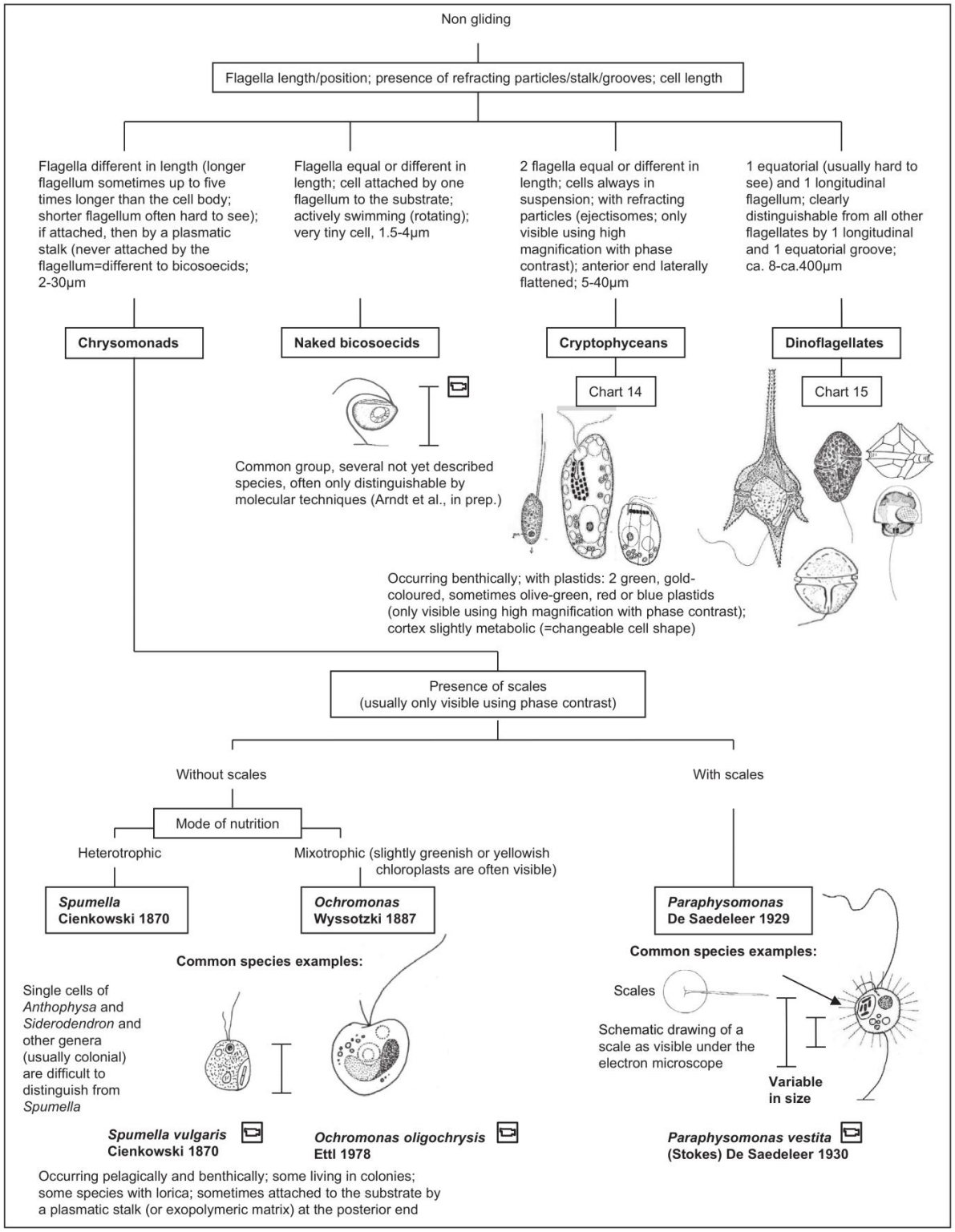
Non gliding
Flagella length/position; presence of refracting particles/stalk/grooves; cell length
Flagella different in length (longer flagellum sometimes up to five times longer than the cell body; shorter flagellum often hard to see); if attached, then by a plasmatic stalk (never attached by the flagellum=different to bicosoecids; 2-30µm
Flagella equal or different in length; cell attached by one flagellum to the substrate; actively swimming (rotating); very tiny cell, 1.5-4µm
2 flagella equal or different in length; cells always in suspension; with refracting particles (ejectisomes; only visible using high magnification with phase contrast); anterior end laterally flattened; 5-40µm
1 equatorial (usually hard to see) and 1 longitudinal flagellum; clearly distinguishable from all other flagellates by 1 longitudinal and 1 equatorial groove; ca. 8-ca.400µm
Chrysomonads
Naked bicosoecids
Cryptophyceans
Dinoflagellates
Chart 14
Chart 15
Common group, several not yet described species, often only distinguishable by molecular techniques (Arndt et al., in prep.)
Occurring benthically; with plastids: 2 green, gold-coloured, sometimes olive-green, red or blue plastids (only visible using high magnification with phase contrast); cortex slightly metabolic (=changeable cell shape)
Presence of scales (usually only visible using phase contrast)
Without scales
With scales
Mode of nutrition
Heterotrophic
Mixotrophic (slightly greenish or yellowish chloroplasts are often visible)
Spumella Cienkowski 1870
Ochromonas Wyssotzki 1887
Paraphysomonas De Saedeleer 1929
Common species examples:
Common species examples:
Single cells of Anthophysa and Siderodendron and other genera (usually colonial) are difficult to distinguish from Spumella
Scales
Schematic drawing of a scale as visible under the electron microscope
Variable in size
Spumella vulgaris Cienkowski 1870
Ochromonas oligochrysis Ettl 1978
Paraphysomonas vestita (Stokes) De Saedeleer 1930
Occurring pelagically and benthically; some living in colonies; some species with lorica; sometimes attached to the substrate by a plasmatic stalk (or exopolymeric matrix) at the posterior end

Chart 4.

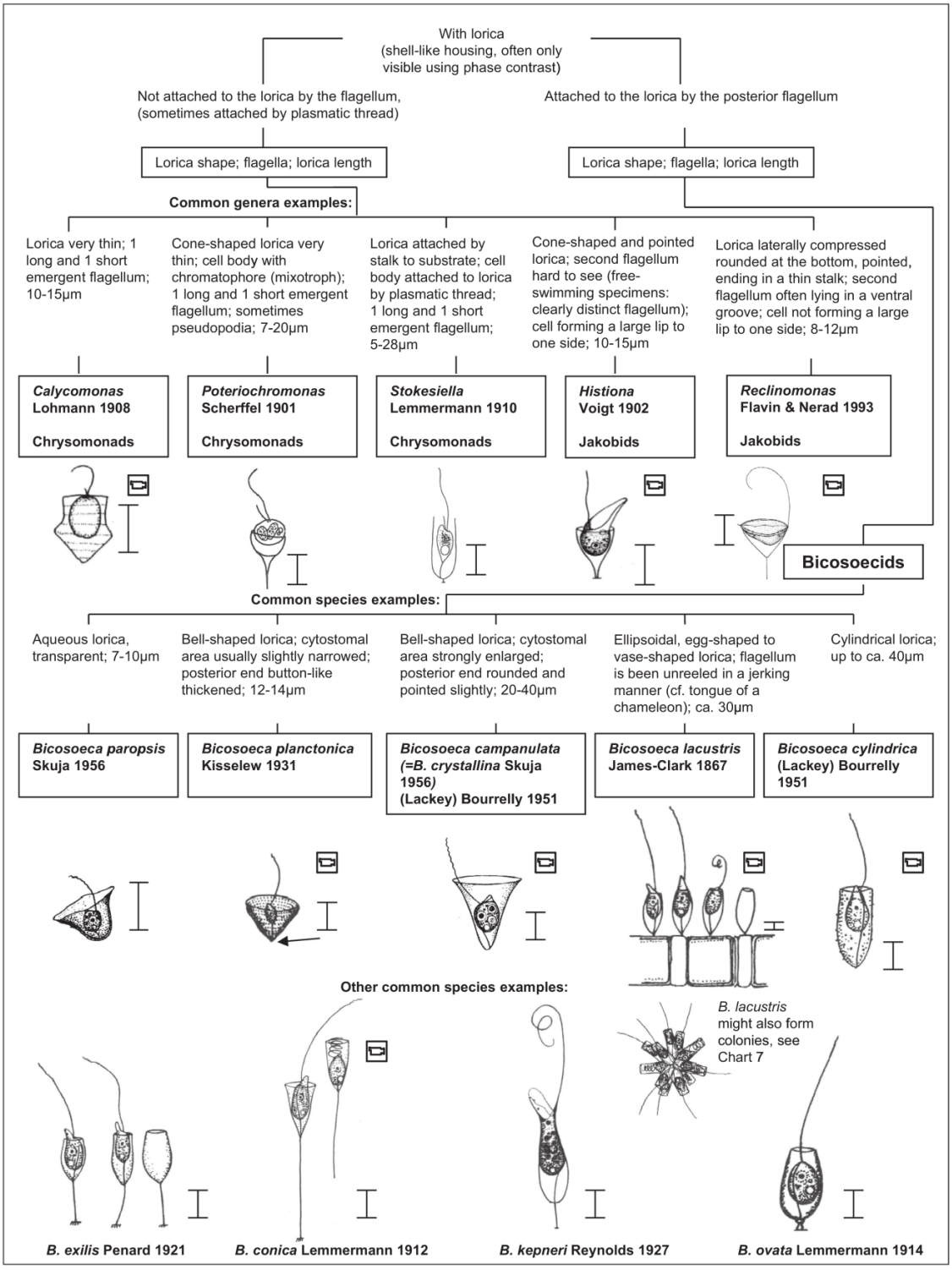
With lorica
(shell-like housing, often only visible using phase contrast)
Not attached to the lorica by the flagellum, (sometimes attached by plasmatic thread)
Attached to the lorica by the posterior flagellum
Lorica shape; flagella; lorica length
Lorica shape; flagella; lorica length
Common genera examples:
Lorica very thin; 1 long and 1 short emergent flagellum; 10-15µm
Cone-shaped lorica very thin; cell body with chromatophore (mixotroph); 1 long and 1 short emergent flagellum; sometimes pseudopodia; 7-20µm
Lorica attached by stalk to substrate; cell body attached to lorica by plasmatic thread; 1 long and 1 short emergent flagellum; 5-28µm
Cone-shaped and pointed lorica; second flagellum hard to see (free-swimming specimens: clearly distinct flagellum); cell forming a large lip to one side; 10-15µm
Lorica laterally compressed rounded at the bottom, pointed, ending in a thin stalk; second flagellum often lying in a ventral groove; cell not forming a large lip to one side; 8-12µm
Calycomonas Lohmann 1908
Chrysomonads
Poteriochromonas Scherffel 1901
Chrysomonads
Stokesiella Lemmermann 1910
Chrysomonads
Histiona Voigt 1902
Jakobids
Reclinomonas Flavin & Nerad 1993
Jakobids
Bicosoecids
Common species examples:
Aqueous lorica, transparent; 7-10µm
Bell-shaped lorica; cytostomal area usually slightly narrowed; posterior end button-like thickened; 12-14µm
Bell-shaped lorica; cytostomal area strongly enlarged; posterior end rounded and pointed slightly; 20-40µm
Ellipsoidal, egg-shaped to vase-shaped lorica; flagellum is been unreeled in a jerking manner (cf. tongue of a chameleon); ca. 30µm
Cylindrical lorica; up to ca. 40µm
Bicosoeca paropsis Skuja 1956
Bicosoeca planctonica Kisselew 1931
Bicosoeca campanulata (=B. crystallina Skuja 1956) (Lackey) Bourrelly 1951
Bicosoeca lacustris James-Clark 1867
Bicosoeca cylindrica (Lackey) Bourrelly 1951
Other common species examples:
B. lacustris might also form colonies, see Chart 7
B. exilis Penard 1921
B. conica Lemmermann 1912
B. kepneri Reynolds 1927
B. ovata Lemmermann 1914

Chart 5.

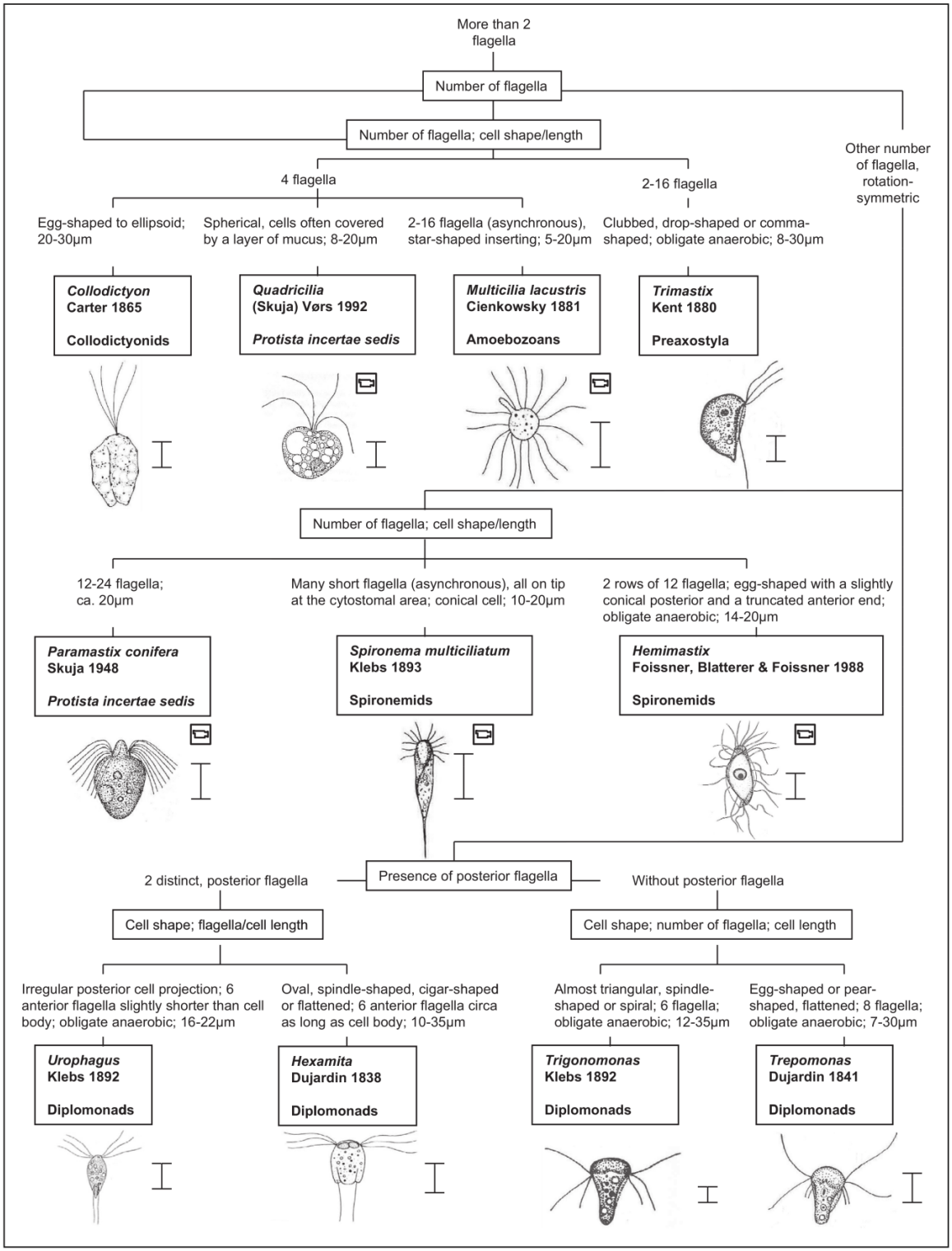
More than 2 flagella
Number of flagella
Number of flagella; cell shape/length
Other number of flagella, rotation-symmetric
4 flagella
2-16 flagella
Egg-shaped to ellipsoid; 20-30µm
Spherical, cells often covered by a layer of mucus; 8-20µm
2-16 flagella (asynchronous), star-shaped inserting; 5-20µm
Clubbed, drop-shaped or comma-shaped; obligate anaerobic; 8-30µm
Collodictyon Carter 1865 Collodictyonids
Quadricilia (Skuja) Vørs 1992 Protista incertae sedis
Multicilia lacustris Cienkowsky 1881 Amoebozoans
Trimastix Kent 1880 Preaxostyla
Number of flagella; cell shape/length
12-24 flagella; ca. 20µm
Many short flagella (asynchronous), all on tip at the cytostomal area; conical cell; 10-20µm
2 rows of 12 flagella; egg-shaped with a slightly conical posterior and a truncated anterior end; obligate anaerobic; 14-20µm
Paramastix conifera Skuja 1948 Protista incertae sedis
Spironema multiciliatum Klebs 1893 Spironemids
Hemimastix Foissner, Blatterer & Foissner 1988 Spironemids
Presence of posterior flagella
2 distinct, posterior flagella
Without posterior flagella
Cell shape; flagella/cell length
Cell shape; number of flagella; cell length
Irregular posterior cell projection; 6 anterior flagella slightly shorter than cell body; obligate anaerobic; 16-22µm
Oval, spindle-shaped, cigar-shaped or flattened; 6 anterior flagella circa as long as cell body; 10-35µm
Almost triangular, spindle-shaped or spiral; 6 flagella; obligate anaerobic; 12-35µm
Egg-shaped or pear-shaped, flattened; 8 flagella; obligate anaerobic; 7-30µm
Urophagus Klebs 1892 Diplomonads
Hexamita Dujardin 1838 Diplomonads
Trigonomonas Klebs 1892 Diplomonads
Trepomonas Dujardin 1841 Diplomonads

Chart 6.

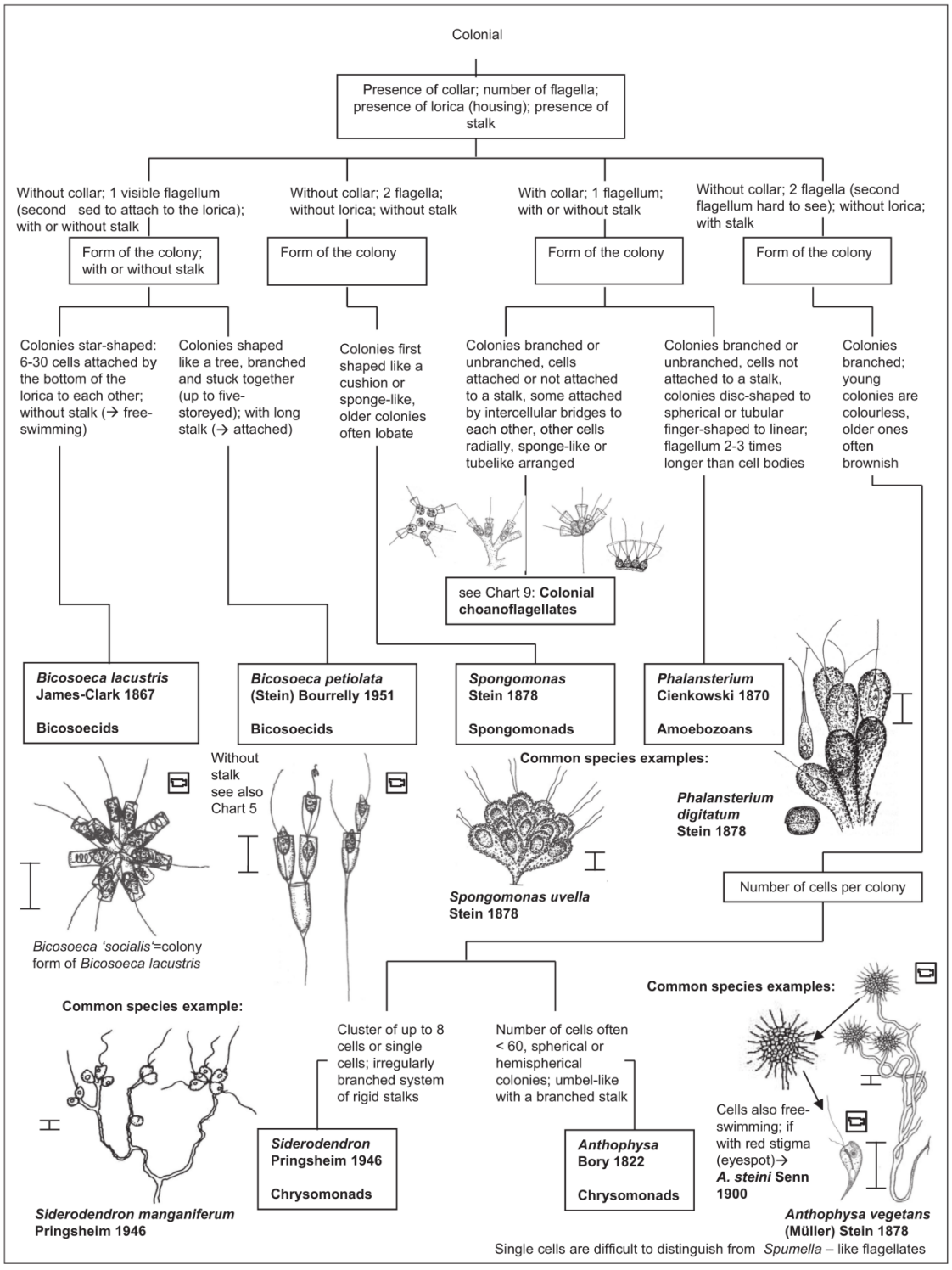
Colonial
Presence of collar; number of flagella; presence of lorica (housing); presence of stalk
Without collar; 1 visible flagellum (second sed to attach to the lorica); with or without stalk
Without collar; 2 flagella; without lorica; without stalk
With collar; 1 flagellum; with or without stalk
Without collar; 2 flagella (second flagellum hard to see); without lorica; with stalk
Form of the colony; with or without stalk
Form of the colony
Form of the colony
Form of the colony
Colonies star-shaped: 6-30 cells attached by the bottom of the lorica to each other; without stalk (→ free-swimming)
Colonies shaped like a tree, branched and stuck together (up to five-storeyed); with long stalk (→ attached)
Colonies first shaped like a cushion or sponge-like, older colonies often lobate
Colonies branched or unbranched, cells attached or not attached to a stalk, some attached by intercellular bridges to each other, other cells radially, sponge-like or tubelike arranged
Colonies branched or unbranched, cells not attached to a stalk, colonies disc-shaped to spherical or tubular finger-shaped to linear; flagellum 2-3 times longer than cell bodies
Colonies branched; young colonies are colourless, older ones often brownish
see Chart 9: Colonial choanoflagellates
Bicosoeca lacustris James-Clark 1867
Bicosoecids
Bicosoeca petiolata (Stein) Bourrelly 1951
Bicosoecids
Spongomonas Stein 1878
Spongomonads
Phalansterium Cienkowski 1870
Amoebozoans
Without stalk see also Chart 5
Common species examples:
Phalansterium digitatum Stein 1878
Spongomonas uvella Stein 1878
Bicosoeca 'socialis'=colony form of Bicosoeca lacustris
Number of cells per colony
Common species example:
Common species examples:
Cluster of up to 8 cells or single cells; irregularly branched system of rigid stalks
Number of cells often < 60, spherical or hemispherical colonies; umbel-like with a branched stalk
Siderodendron Pringsheim 1946
Chrysomonads
Anthophysa Bory 1822
Chrysomonads
Cells also free-swimming; if with red stigma (eyespot)→ A. steini Senn 1900
Siderodendron manganiferum Pringsheim 1946
Anthophysa vegetans (Müller) Stein 1878
Single cells are difficult to distinguish from Spumella – like flagellates

Chart 7.

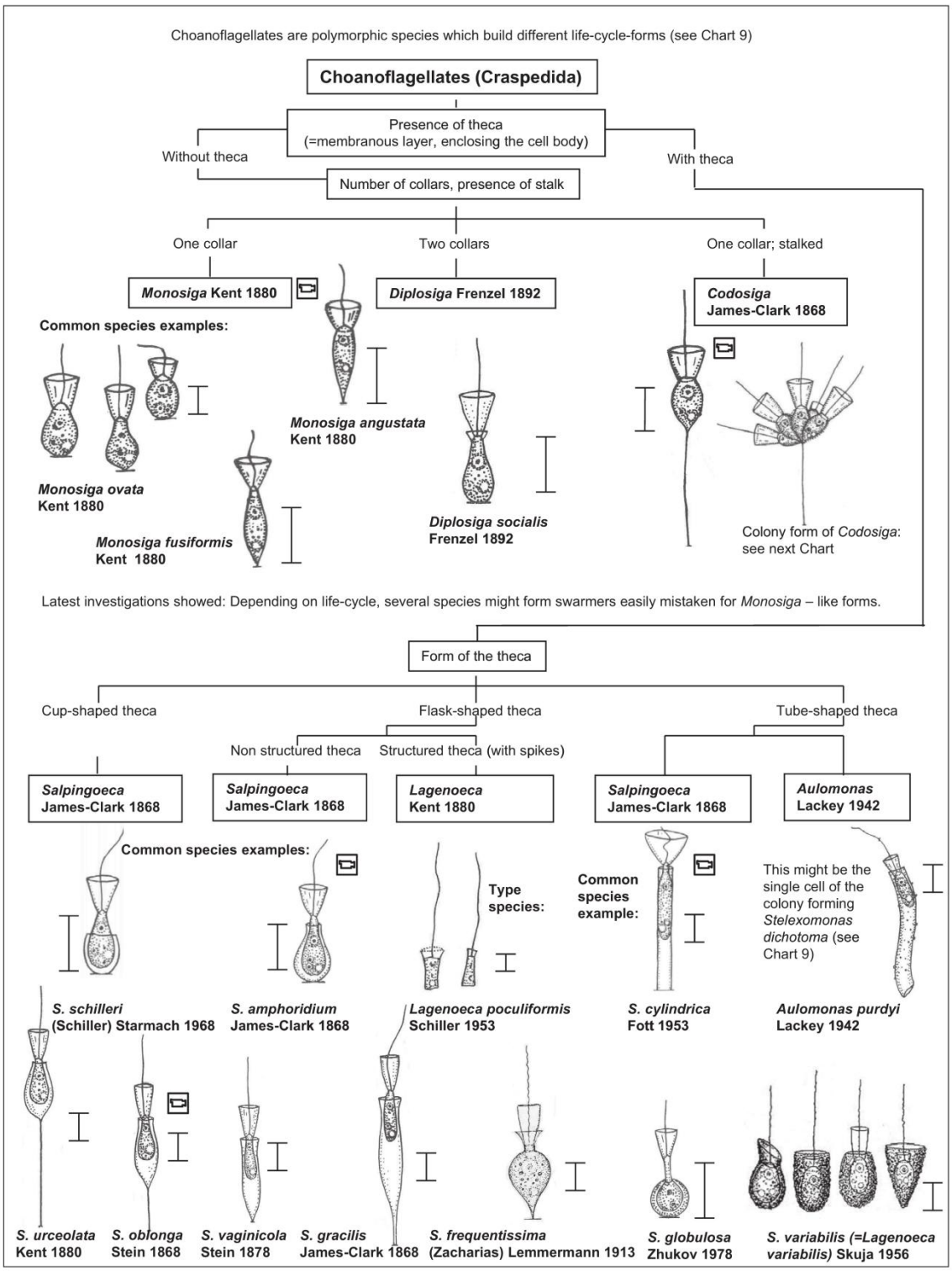
Choanoflagellates are polymorphic species which build different life-cycle-forms (see Chart 9)
Choanoflagellates (Craspedida)
Presence of theca
(=membranous layer, enclosing the cell body)
Without theca
With theca
Number of collars, presence of stalk
One collar
Two collars
One collar; stalked
Monosiga Kent 1880
Diplosiga Frenzel 1892
Codosiga
James-Clark 1868
Common species examples:
Monosiga ovata
Kent 1880
Monosiga fusiformis
Kent 1880
Monosiga angustata
Kent 1880
Diplosiga socialis
Frenzel 1892
Colony form of Codosiga:
see next Chart
Latest investigations showed: Depending on life-cycle, several species might form swarmers easily mistaken for Monosiga – like forms.
Form of the theca
Cup-shaped theca
Flask-shaped theca
Tube-shaped theca
Non structured theca
Structured theca (with spikes)
Salpingoeca
James-Clark 1868
Salpingoeca
James-Clark 1868
Lagenoeca
Kent 1880
Salpingoeca
James-Clark 1868
Aulomonas
Lackey 1942
Common species examples:
Type
species:
Common
species
example:
This might be the
single cell of the
colony forming
Stelexomonas
dichotoma (see
Chart 9)
S. schilleri
(Schiller) Starmach 1968
S. amphoridium
James-Clark 1868
Lagenoeca poculiformis
Schiller 1953
S. cylindrica
Fott 1953
Aulomonas purdyi
Lackey 1942
S. urceolata
Kent 1880
S. oblonga
Stein 1868
S. vaginicola
Stein 1878
S. gracilis
James-Clark 1868
S. frequentissima
(Zacharias) Lemmermann 1913
S. globulosa
Zhukov 1978
S. variabilis (=Lagenoeca
variabilis) Skuja 1956

Chart 8.

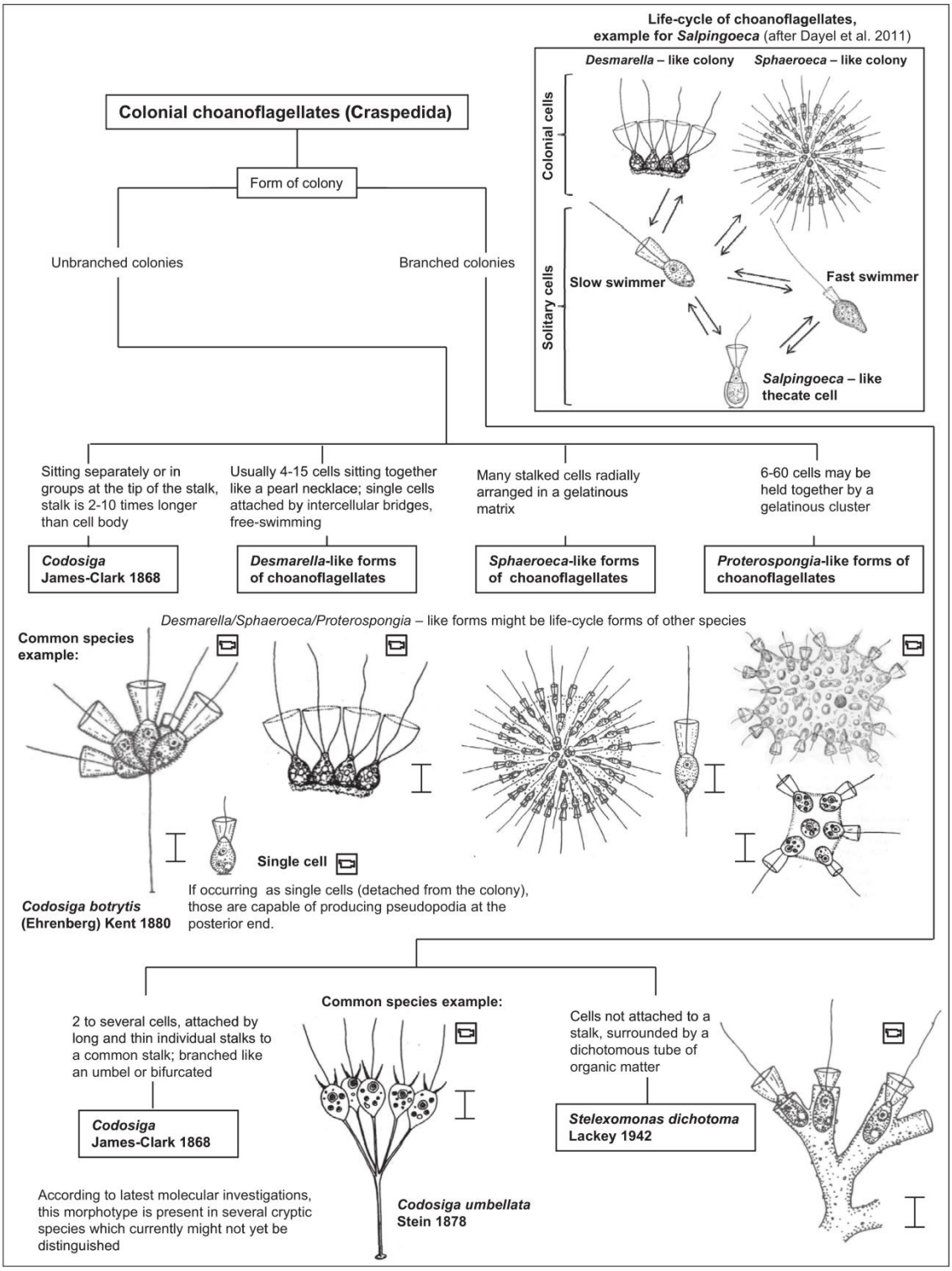
Life-cycle of choanoflagellates, example for Salpingoeca (after Dayel et al. 2011)
Desmarella – like colony
Sphaeroeca – like colony
Colonial cells
Solitary cells
Slow swimmer
Fast swimmer
Salpingoeca – like thecate cell
Colonial choanoflagellates (Craspedida)
Form of colony
Unbranched colonies
Branched colonies
Sitting separately or in groups at the tip of the stalk, stalk is 2-10 times longer than cell body
Usually 4-15 cells sitting together like a pearl necklace; single cells attached by intercellular bridges, free-swimming
Many stalked cells radially arranged in a gelatinous matrix
6-60 cells may be held together by a gelatinous cluster
Codosiga James-Clark 1868
Desmarella-like forms of choanoflagellates
Sphaeroeca-like forms of choanoflagellates
Proterospongia-like forms of choanoflagellates
Desmarella/Sphaeroeca/Proterospongia – like forms might be life-cycle forms of other species
Common species example:
Single cell
Codosiga botrytis (Ehrenberg) Kent 1880
If occurring as single cells (detached from the colony), those are capable of producing pseudopodia at the posterior end.
Common species example:
2 to several cells, attached by long and thin individual stalks to a common stalk; branched like an umbel or bifurcated
Codosiga James-Clark 1868
According to latest molecular investigations, this morphotype is present in several cryptic species which currently might not yet be distinguished
Codosiga umbellata Stein 1878
Cells not attached to a stalk, surrounded by a dichotomous tube of organic matter
Stelexomonas dichotoma Lackey 1942

Chart 9.

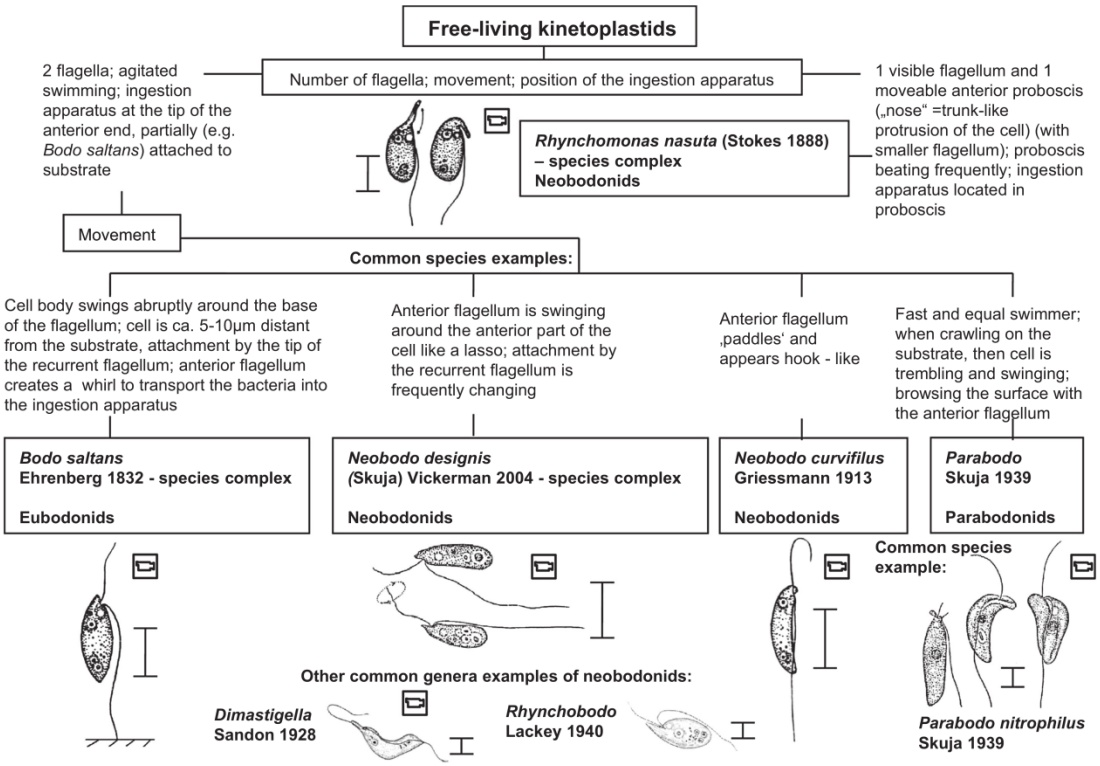

Chart 10.

(e.g. Pawlowski et al. 2011; Stoeck et al. 2010; and Domonell et al., unpubl. observ.). However, their application still bears a lot of problems concerning the following points: 1) many specific primers are necessary to cover the whole HF diversity due to the wide molecular diversity of HF (some groups will be overlooked), 2) active and inactive forms are difficult to differentiate, 3) databases are incomplete (Will and Rubinoff 2004) and contain high numbers of errors (Prosdocimi et al. 2013). Quantitative morphotype studies will provide helpful information to increase reliability of databases and offer important knowledge on the ecology and evolution of HF groups significant in ecosystems (Pawlowski et al. 2012). We would like to increase the resolution of these quantitative morphotype studies by this taxonomic guide.

How to Use the Guide

The present guide is intended to help also unexperienced researchers to identify HF morphotypes, although it is recommended to have a general knowledge of HF (recommended literature: e.g. Hausmann et al. 2003; Leadbeater and Green 2000; Patterson and Larsen 1991). The systematic position of flagellate groups considered in the guide is given in Figure 1 following the recent revision by Adl et al. (2012). Following the idea of Foissner and Berger (1996) with regard to ciliates, we designed a polytomous guide. The first Chart contains a general overview of the most important characters of the main groups of protists. The user is led to a "fast-track" regarding the main groups of HF (Charts 1–15) by schematic drawings with arrows pointing to the important characters. If identification is already sufficient the user is led to the special Chart of the organism. If not, he should proceed to Chart 2. The user is led from obvious characters (e.g. colonial or single cells, number of flagella) to more detailed descriptions. Features of organisms which are used to discriminate between different taxa are set within boxes while the discriminator for the subsequent path of identification is shown without boxes. Figures are added to the descriptions of characters to accelerate and increase the chance of identification. All scale bars in the guide indicate

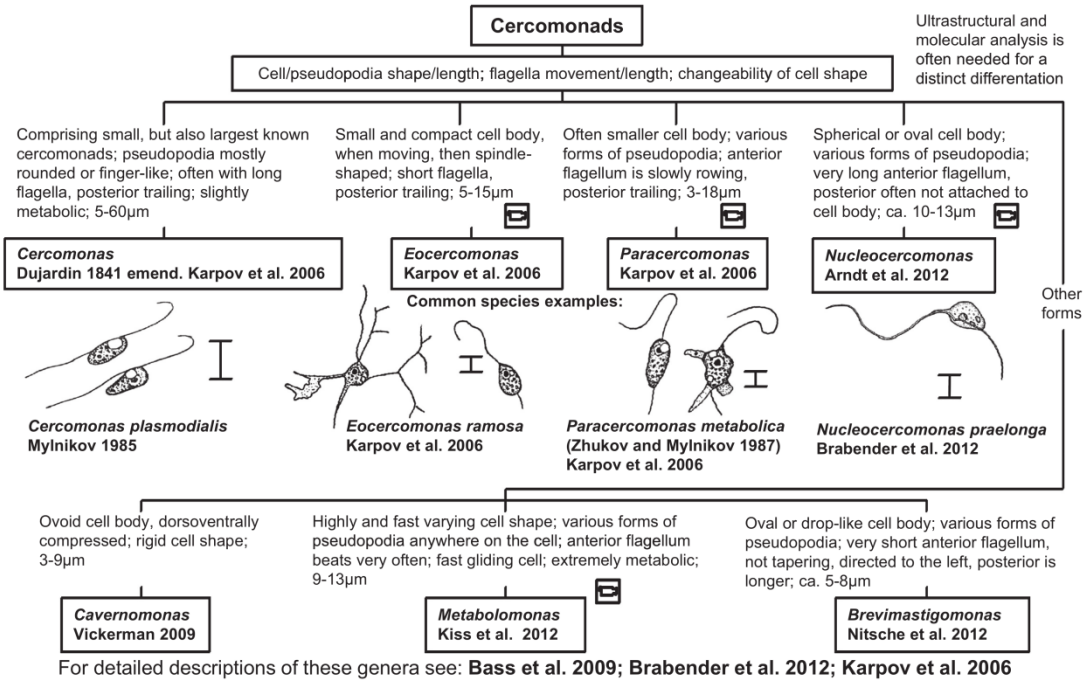

Chart 11.

10 µm. The guide leads only to the species or genus morphotype. It is widely accepted that many protistan morphotypes contain a few up to hundreds of genotypes (typical examples are the common species *Neobodo designis* (Scheckenbach et al. 2006) or *Codosiga botrytis* (Stoupin et al. 2012)), see above. If video sequences (Supplementary Material) are available for the organisms, they are marked by a video icon (🖃). Some additional literature is given on the Charts (Cavalier-Smith and Chao 2010; Bass et al. 2009; Brabender et al. 2012; Karpov et al. 2006). Due to the fact that major forms of heterotrophic flagellates are ubiquitous in marine and freshwater environments, this guide may also be useful in parts for an orientation regarding flagellate groups in brackish water systems.

Methods

The guide is based on an analysis using the classical living-droplet method by phase contrast/DIC light microscopy (e.g. Arndt and Mathes 1991; Gasol 1993; Massana and Güde 1991). This method is reliable to detect morphological and behavioural characteristics of even very small HF regarding their main taxonomic groups – in contrast to hidden characteristics within samples treated by fixatives. Pelagic flagellates (undiluted samples) and benthic flagellates (diluted by a factor of 5- >20 with filtered water) can be studied in droplets of 5-20 µl on a prepared microscope slide or an Utermöhl chamber (Utermöhl 1958; HydroBios GmbH, Kiel, Germany; Fig. 2). The HF composition has to be analysed within one hour after sampling by means of a phase contrast microscope equipped with high resolution video-recording which is helpful for further and later identification. 20x, 40x objectives are helpful for rough morphological observations and quantitative counts. Individual samples can be analysed within a few minutes (particularly critical for pelagic samples). The use of a 63x long distance objective or water immersion objectives (63x and 100x; with a long working distance) are recommended for morphotype identifications. Phase contrast equipment is mostly indispensible to analyse position and movement of flagella, the presence of collar, lorica, or stalk of some flagellates. Differential interference contrast (DIC) equipment might additionally help to identify cell structures such as paramylon, ingestion apparatus, vacuoles or extrusomes.

A realistic compromise should be a combination of counts by the living-droplet method with counts of chemically fixed samples and analysis by epifluorescence microscopy. The following fixatives are commonly in use: 2% glutaraldehyde (e.g. Caron 1983; Choi and Stoecker 1989), 2% formaldehyde (e.g. Porter and Feig 1980; Sherr et al. 1989), buffered formaldehyde (e.g. Børsheim and Bratbak 1987; Sherr and Sherr 1983) and 0.5% acidic Lugol' solution (10 g I_2, 20 g KI, 10 g sodium acetate in 140 ml aq. dest.) plus 3% formaldehyde (e.g. Sherr et al. 1989).

The fixed samples need to be kept at 4 °C in the dark until staining. Fluorescent dyes (e.g. DAPI (= 4′6-diamidino-2-phenylindole, Sigma Lot 47H4107, Porter and Feig 1980) are suitable for staining. After staining the sample should be filtered onto a 0.2-µm membrane filter using vacuum not exceeding 150 mm Hg. For an even distribution of the particles, another

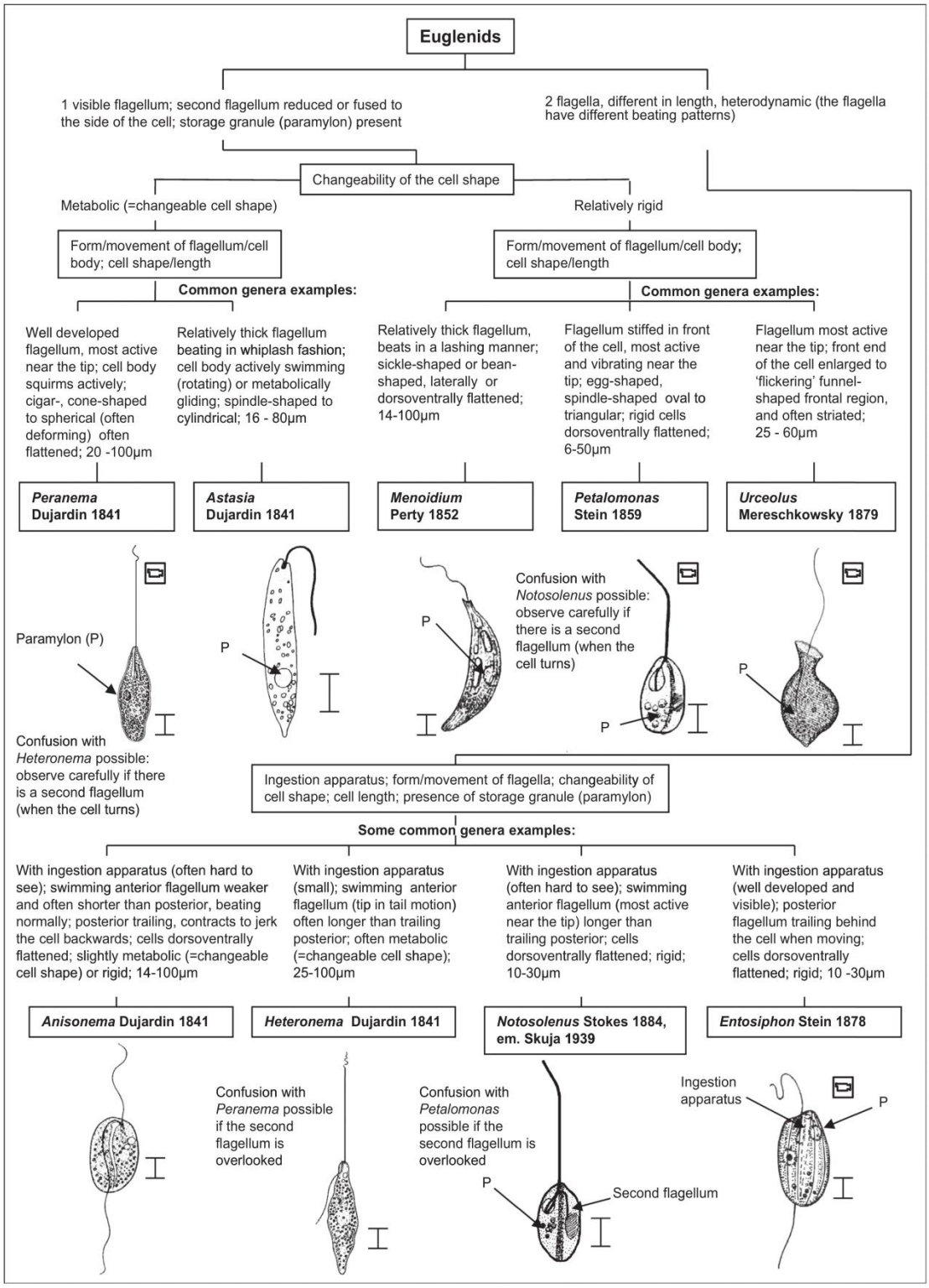
Euglenids
1 visible flagellum; second flagellum reduced or fused to the side of the cell; storage granule (paramylon) present
2 flagella, different in length, heterodynamic (the flagella have different beating patterns)
Changeability of the cell shape
Metabolic (=changeable cell shape)
Relatively rigid
Form/movement of flagellum/cell body; cell shape/length
Form/movement of flagellum/cell body; cell shape/length
Common genera examples:
Common genera examples:
Well developed flagellum, most active near the tip; cell body squirms actively; cigar-, cone-shaped to spherical (often deforming) often flattened; 20 -100µm
Relatively thick flagellum beating in whiplash fashion; cell body actively swimming (rotating) or metabolically gliding; spindle-shaped to cylindrical; 16 - 80µm
Relatively thick flagellum, beats in a lashing manner; sickle-shaped or bean-shaped, laterally or dorsoventrally flattened; 14-100µm
Flagellum stiffed in front of the cell, most active and vibrating near the tip; egg-shaped, spindle-shaped oval to triangular; rigid cells dorsoventrally flattened; 6-50µm
Flagellum most active near the tip; front end of the cell enlarged to 'flickering' funnel-shaped frontal region, and often striated; 25 - 60µm
Peranema Dujardin 1841
Astasia Dujardin 1841
Menoidium Perty 1852
Petalomonas Stein 1859
Urceolus Mereschkowsky 1879
Paramylon (P)
P
P
P
Confusion with Notosolenus possible: observe carefully if there is a second flagellum (when the cell turns)
P
Confusion with Heteronema possible: observe carefully if there is a second flagellum (when the cell turns)
Ingestion apparatus; form/movement of flagella; changeability of cell shape; cell length; presence of storage granule (paramylon)
Some common genera examples:
With ingestion apparatus (often hard to see); swimming anterior flagellum weaker and often shorter than posterior, beating normally; posterior trailing, contracts to jerk the cell backwards; cells dorsoventrally flattened; slightly metabolic (=changeable cell shape) or rigid; 14-100µm
With ingestion apparatus (small); swimming anterior flagellum (tip in tail motion) often longer than trailing posterior; often metabolic (=changeable cell shape); 25-100µm
With ingestion apparatus (often hard to see); swimming anterior flagellum (most active near the tip) longer than trailing posterior; cells dorsoventrally flattened; rigid; 10-30µm
With ingestion apparatus (well developed and visible); posterior flagellum trailing behind the cell when moving; cells dorsoventrally flattened; rigid; 10 -30µm
Anisonema Dujardin 1841
Heteronema Dujardin 1841
Notosolenus Stokes 1884, em. Skuja 1939
Entosiphon Stein 1878
Confusion with Peranema possible if the second flagellum is overlooked
Confusion with Petalomonas possible if the second flagellum is overlooked
P
Second flagellum
Ingestion apparatus
P

Chart 12.

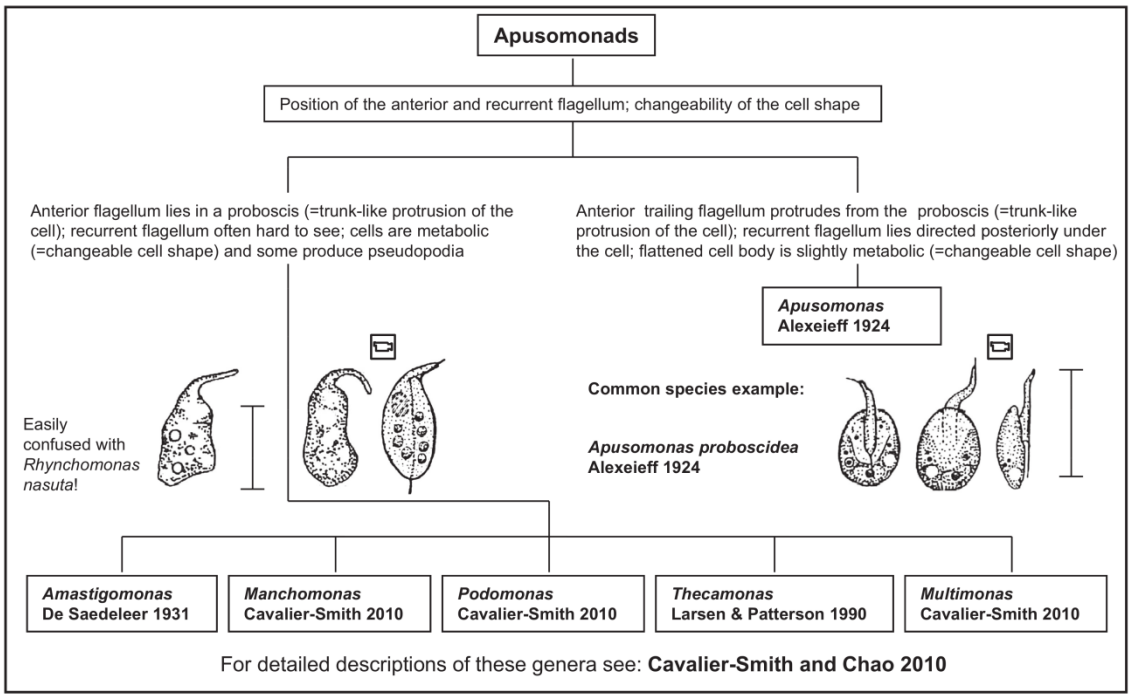

Chart 13.

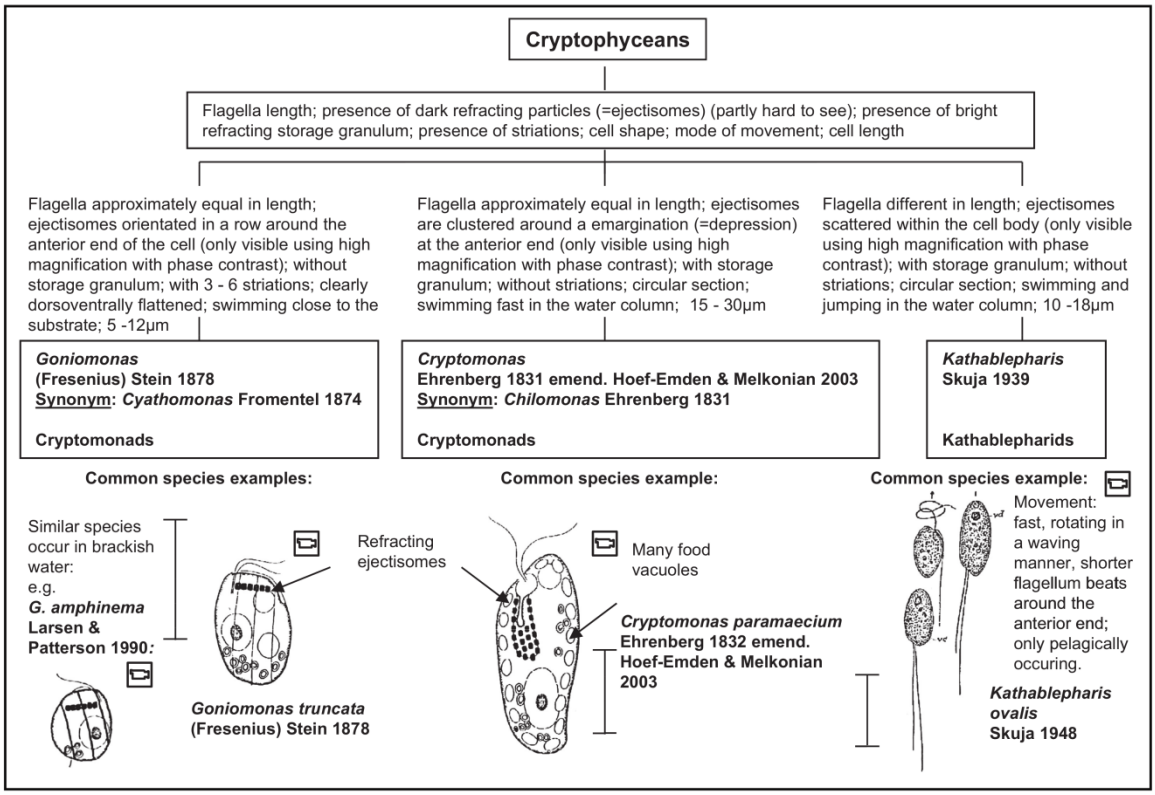

Chart 14.

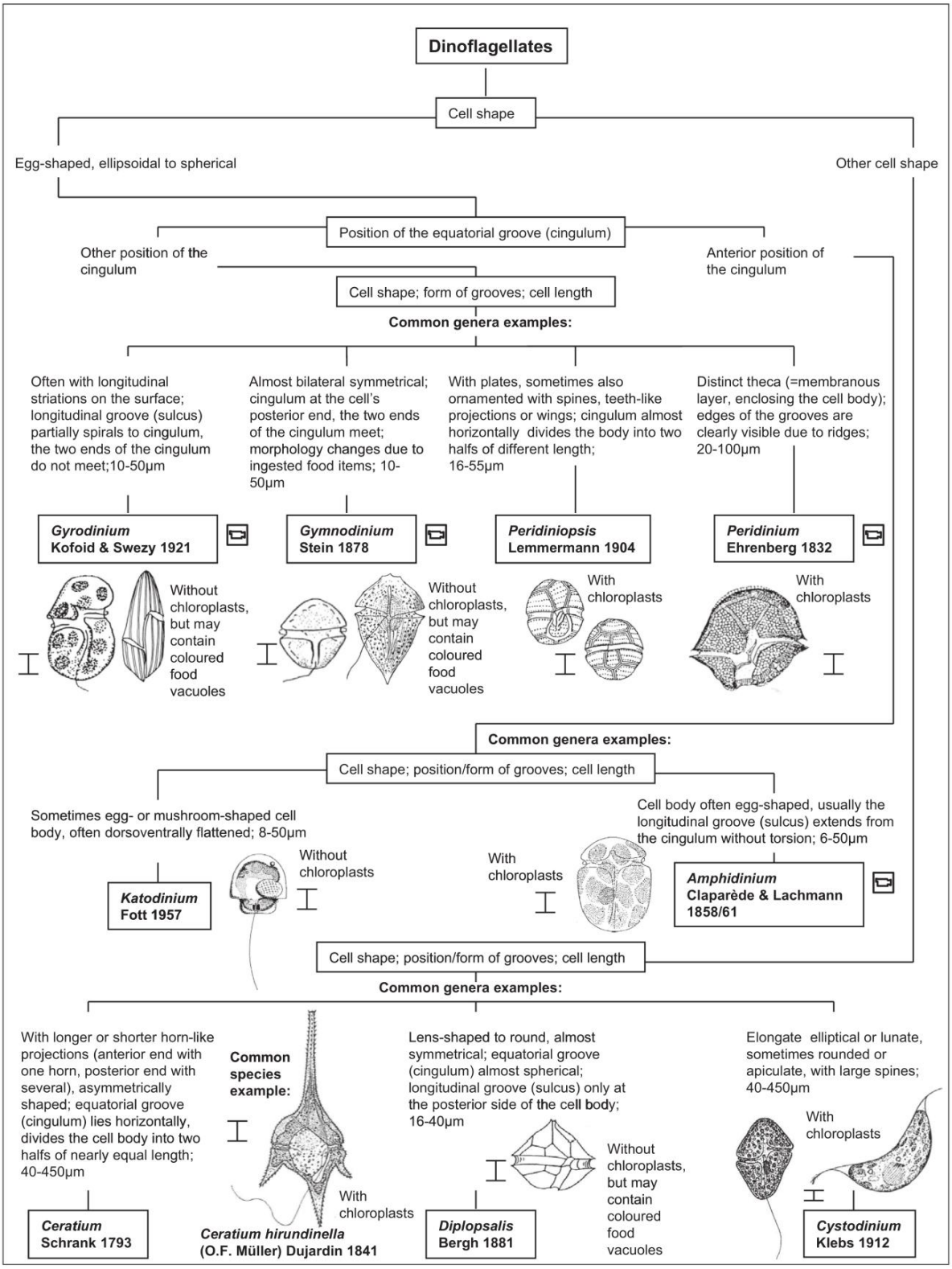
Dinoflagellates
Cell shape
Egg-shaped, ellipsoidal to spherical
Other cell shape
Position of the equatorial groove (cingulum)
Other position of the cingulum
Anterior position of the cingulum
Cell shape; form of grooves; cell length
Common genera examples:
Often with longitudinal striations on the surface; longitudinal groove (sulcus) partially spirals to cingulum, the two ends of the cingulum do not meet;10-50µm
Almost bilateral symmetrical; cingulum at the cell's posterior end, the two ends of the cingulum meet; morphology changes due to ingested food items; 10-50µm
With plates, sometimes also ornamented with spines, teeth-like projections or wings; cingulum almost horizontally divides the body into two halfs of different length; 16-55µm
Distinct theca (=membranous layer, enclosing the cell body); edges of the grooves are clearly visible due to ridges; 20-100µm
Gyrodinium Kofoid & Swezy 1921
Gymnodinium Stein 1878
Peridiniopsis Lemmermann 1904
Peridinium Ehrenberg 1832
Without chloroplasts, but may contain coloured food vacuoles
Without chloroplasts, but may contain coloured food vacuoles
With chloroplasts
With chloroplasts
Common genera examples:
Cell shape; position/form of grooves; cell length
Sometimes egg- or mushroom-shaped cell body, often dorsoventrally flattened; 8-50µm
Cell body often egg-shaped, usually the longitudinal groove (sulcus) extends from the cingulum without torsion; 6-50µm
Katodinium Fott 1957
Without chloroplasts
With chloroplasts
Amphidinium Claparède & Lachmann 1858/61
Cell shape; position/form of grooves; cell length
Common genera examples:
With longer or shorter horn-like projections (anterior end with one horn, posterior end with several), asymmetrically shaped; equatorial groove (cingulum) lies horizontally, divides the cell body into two halfs of nearly equal length; 40-450µm
Common species example:
With chloroplasts
Ceratium hirundinella (O.F. Müller) Dujardin 1841
Lens-shaped to round, almost symmetrical; equatorial groove (cingulum) almost spherical; longitudinal groove (sulcus) only at the posterior side of the cell body; 16-40µm
Without chloroplasts, but may contain coloured food vacuoles
Elongate elliptical or lunate, sometimes rounded or apiculate, with large spines; 40-450µm
With chloroplasts
Ceratium Schrank 1793
Diplopsalis Bergh 1881
Cystodinium Klebs 1912

Chart 15.

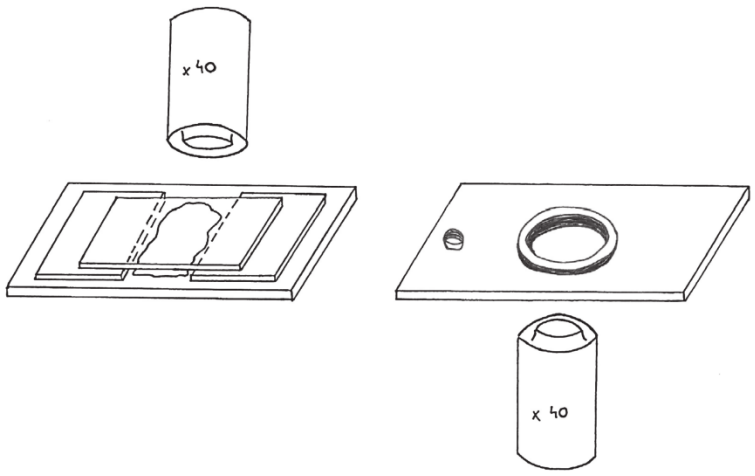

Figure 2. Schematic drawing of some helpful counting chambers. a) Simple microchamber: Two cover slips are placed on the slide (each on every end). A 10 μl droplet is placed between them and a third cover slip above the droplet and the two slips. b) Utermöhl chamber for counting living flagellates or sedimented, chemically fixed samples.

membrane (0.6 μm pore size) may be placed underneath the filter. The filters are generally stored deep-frozen at –20 °C until examination by an epifluorescence microscope.

It is additionally recommended to establish cultures of HF isolated from the samples (e.g. simply by adding autoclaved wheat grains etc. as a carbon source for food bacteria to parts of raw samples). This should aid in morphotype identification of bacterivorous forms and to check whether the determined morphotypes are life-cycle forms (swarmer, colony etc.) of other types, as it is often the case with e.g. choanoflagellates where extrinsic factors can induce the formation of multicellular forms (Alegado et al. 2012; Dayel et al. 2011). However, it has to be considered that all cultivation and fixation methods (and also molecular methods) can be very selective. According to our own experience, the percentage of live-counted organisms appearing in cultures ranges between 1 and 30 per cent.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.protis.2013.08.003.

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Supplementary Material

All video sequences can be found at http://dx.doi.org/10.1016/j.protis.2013.08.003.

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Ancyromonadids (Fig. 2): original.

Ancyromonas: Patterson and Zölffel 1991, p. 433.

Anisonema: Streble and Krauter 2008, p. 159.

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Eocercomonas ramosa: Karpov et al. 2006, p. 132.

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Gymnoeca: original.

Gyrodinium (Overview chart 1+ Fig. 2): original.

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Gyrodinium: Popovsky and Pfister 1990, p. 137.

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Salpingoeca amphoridium (flask-shaped theca): Zhukov 1993, p. 134.

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Salpingoeca frequentissima: Skuja 1956, Tab. LIV.

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Salpingoeca schilleri (cup-shaped theca): Zhukov 1993, p. 134.

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Salpingoeca vaginicola (tube-shaped theca): Zhukov 1993, p. 134.

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Sphaeroeca volvox: Zhukov 1993, p. 133.

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Spironema (Overview chart 1 + Fig. 2): original.

Spironema multiciliatum: Patterson and Zölffel 1991, p. 460.

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Spumella (Overview chart 1+ Fig. 2): original.

Spumella vulgaris: Starmach 1985, p. 191.

Spumella vulgaris (without second flagellum): after Starmach 1985, p. 191.

Stelexomonas dichotoma: Zhukov 1993, p. 135.

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Trimastix (Fig. 2): original.

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Chapter 2

Extended phylogeny of the Craspedida (Choanomonada)

Abstract

Currently choanoflagellates are classified into two distinct orders: loricate Acanthoecida and non-loricate Craspedida. The morphologically based taxonomy of the order Craspedida is in need of a revision due to its controversial, paraphyletic and not consistent systematics and nomenclature. In this study, we add molecular data (SSU and parts of the LSU rDNA) of six new Craspedida species isolated from saline, brackish and freshwater habitats to the existing knowledge. Four of these six organisms could be described as new species: *Paramonosiga thecata*, *"Salpingoeca" euryoecia*, *"Salpingoeca" ventriosa*, *"Sphaeroeca" leprechaunica,* whereas two are assigned to previous morphologically described species: *"Salpingoeca" fusiformis* Saville Kent (1880) and *"Salpingoeca" longipes* Saville Kent (1880). *Paramonosiga* is established as a new genus of the Craspedida based on its phylogenetic position. Extending the dataset by six additional sequences shows that the craspedid taxonomy is still unsolved as the type specimen *Salpingoeca gracilis* has not yet been sequenced and hence a clear assignment of the genus *Salpingoeca* is not possible. Trying to assign morphological and ecological data to phylogenetic clades is not successful. We give an improved/emended morphological diagnosis for the two redescribed species and add molecular data for all six species, shedding light on their phylogenetic position.

Introduction

Choanoflagellates (Choanomonada) are small phagotrophic protists ubiquitously distributed in all aquatic habitats and even found in dry soil. They possess a single apical flagellum surrounded by a collar of microvilli. Within the group of Opisthokonta, choanoflagellates form the sister group to the Metazoa and are therefore of great interest as models to understand early evolution of multicellularity in metazoans (Carr et al. 2008; Cavalier-Smith and Chao 2003, King et al. 2008; Medina et al. 2003; Richter and King 2013; Ruiz-Trillo et al. 2008; Steenkamp et al. 2006). Apart from that, choanoflagellates are also of great ecological importance due to their occasional high abundance in many different aquatic environments (e.g. Leadbeater 1974; Nitsche and Arndt 2008; Scheckenbach et al. 2010; Stock et al. 2012; Thomsen et al. 1997; Tong 1997; Wylezich and Jürgens 2011). Despite their relatively small size, they consume large amounts of bacteria and have thus a significant impact on the food web (Arndt et al. 2000; Boenigk and Arndt 2002).

In older taxonomic works (Norris 1965), choanoflagellates are divided into three families: Acanthoecidae (Acanthoecaceae) Norris (1965), Salpingoecidae Saville Kent (1880), and Codonosigidae Saville Kent (1880). This old classification is mainly based on the presence or absence of an organic cell coating. According to Norris (1965), those species possessing basket-like loricae, consisting of silicified costae, are Acanthoecidae. The family of the Salpingoecidae comprise species having a rigid organic theca surrounding the cell body. In contrast, Codonosigidae are “naked” species without a restrictive covering but with a fine coating (glycocalyx) (see Saville Kent 1880). Representatives of the genus *Codosiga* often possess a microfibrillar stalk when forming multi-headed stalked colonies. Other genera like *Monosiga* and *Desmarella* are able to form chain-like colonies (*Desmarella*) or occur as single cells (*Monosiga*).

Recent molecular data, mainly based on SSU rDNA (ribosomal DNA), support the well-defined phylogeny of the loricate choanoflagellates as comprising two monophyletic families: nudiform Acanthoecidae Norris emend. *sensu* Nitsche et al. (2011) and tectiform Stephanoecidae Leadbeater (2011). These two families are now

classified within the order Acanthoecida Cavalier-Smith (1996). In contrast, the molecular data do not underline the taxonomy of the non-loricate choanoflagellates: The differentiation of the two families, Salpingoecidae and Codonosigidae named by Saville Kent (1880-81), are abandoned by Nitsche et al. (2011). Both are now concentrated in the order Craspedida Cavalier-Smith (1996) as they are clearly not monophyletic, because the Codonosigidae are recovered as polyphyletic lineages within the Salpingoecidae (Carr et al. 2008). Three different forms of theca morphology can be observed within the Craspedida: Flask-shaped (example: *Choanoeca perplexa*, see Leadbeater 1977), cup-shaped (example: *Salpingoeca rosetta*, see Dayel et al. 2011) and tube-shaped forms (example: *Salpingoeca tuba*, see Nitsche et al. 2011).

Unfortunately, most descriptions of choanoflagellate species since the 19th century have only considered morphological features. Taking this into account and the fact that there is cryptic diversity within morphospecies and that there may be a variety of life cycle forms (Stoupin et al. 2012; Dayel et al. 2011), the necessity of a revised craspedid taxonomy is obvious. In this study, the sequencing of the SSU (small subunit) and fragments of the LSU (large subunit) rDNA of six isolates from saline, brackish, and freshwater have revealed new insights into the taxonomy and systematics of the Craspedida.

Material and Methods

Material collection and culturing

Six isolates of craspedid choanoflagellate species were collected from six different sampling sites in Europe, North America and South America in different habitats (Table 1). From each collection site, 500 ml of surface water was taken in a sterile polyethylene bottle. Aliquots were transferred to cell culture flasks (50 ml, Sarstedt, Nümbrecht, Germany). A sterilized wheat grain was provided as nutrition for autochthonous bacterial growth as food source for the choanoflagellates. All freshwater and the one estuarine sample were cultivated using Wright's Chu medium

(Guillard and Lorenzen 1972). For the marine samples, artificial seawater with a salinity of 35 PSU (practical salinity units) was used (per litre deionized water: 28.15 g NaCl, 0.67 g KCl, 5.51 g $MgCl_2 \cdot 6H_2O$, 1.45 g $CaCl_2 \cdot 2H_2O$, 6.92 g $MgSO_4 \cdot 7H_2O$, 0.1 g KNO_3, 0.01 g $K_2HPO_4 \cdot 3H_2O$). All samples were kept at 10°C and a 12/12h day/night cycle. Each week, cultures were examined under light microscopy (Zeiss Axiovert S 100). To obtain clonal cultures, the raw cultures containing choanoflagellates were diluted using the liquid aliquot method (Butler and Rogerson 1995) or using a micromanipulator (Patchman MP2, Eppendorf, Hamburg, Germany) to transfer single cells to culture flasks.

Video microscopy

For the investigation of the morphological structures, we used an inverted light microscope system. Clonal cultures were cultivated in Petri dishes prepared with coverslips fixed at the Petri dish bottom (Petri dishes were blanked out, coverslips used as base). The Petri dishes with the coverslips were observed by a Zeiss Axio Observer with a 100x/1.4 NA oil immersion objective (DIC) and a water immersion condenser. Video images were taken using a black/white analogous Hamamatsu C6489 camera with noise reduction and contrast enhancement by an Allen Video Enhanced Contrast (AVEC) system (Hamamatsu, Argus-20) (for details regarding this and the microscope setup see Stoupin et al. 2012). Videos were analysed frame by frame. For image analysis the programs VirtualDub (www.virtualdub.org), ImageJ (Abramoff et al. 2004), and AviStack (www.avistack.de) were used.

Electron microscopy

For scanning electron microscopy, the samples were fixed at a ratio of one to one with Bouin's fixative and 1% osmium tetroxide (final concentration) at 4°C for 30 min. The Bouin`s fixative contained three parts saturated picric acid and one part buffered formaldehyde (38%) with 2% glacial acetic acid, added immediately before fixation. Glutaraldehyde was added to the final solution to give a final concentration of

0.1-0.2%. Samples remained in the culture flask (Sarstedt 50ml culture flasks) and were dehydrated in an ethanol series comprising 30%, 50%, 60%, 80%, 90%, 96% and pure ethanol. Samples were washed two times with the corresponding ethanol concentration and finally remained for 10 min in each solution. After this procedure, a 50:50 hexamethyldisilazane (HMDS)-ethanol solution was applied for 15 min followed by pure HMDS for 15 min as a substitute for critical point drying (Nitsche and Arndt 2008). Afterwards, the samples were allowed to dry. The bottom of each flask was cut to appropriate size and stuck to a sample holder. SEM samples were sputter coated with a 120Å layer of gold before examination by SEM (FEI Quanta 250 FEG).

Molecular biological analysis

The amplification of the SSU rDNA was carried out using both single-cell and total genomic DNA. The LSU rDNA amplification was achieved using the latter only. Single cells isolated by a micromanipulator (Eppendorf, Hamburg, Germany) were transferred to 15 µl ddH_2O and deep frozen at -80°C for 15 min before PCR (modified after Nitsche and Arndt 2008). We firstly amplified the SSU rDNA fragment using 42F (5'-CTCAARGAYTAAGCCATGCA-3') and 18S-Rev-1 (5'-ACCTACGGAAACCTTGT-TACG-3') primers with a concentration of 0.1mM, using a PCR Mastermix (2x) (Genaxxon Bioscience, Ulm, Germany) for all reactions. The mixture was heated to 96°C for 2min, followed by 32 cycles of 95°C for 30s, 48°C for 30s, 52°C for 30s, and 72°C for 2min, finished by 7 min at 72°C. Reamplification was done using the primers 82F (5'-GAAACTGCGAATGGCTC-3') and 1630R (5'-CGACGGGCGGTGTGACAA-3') with the same amplification steps. The PCR products were purified by the PCR Purification Kit (Bioscience, Jena, Germany) and sequenced for both strands (82F + 1630R).

For the total genomic DNA PCR reactions, DNA extraction was performed using the Quick gDNA^{TM} Mini Prep (Zymo Research Corporation, CA, USA). We sequenced the SSU rDNA using the following primer combinations (0.1mM concentration): 42F + 18S-Rev-1, 82F + 1630R, and 590F (5'- CGGTAATTCCAGCTCCAATAGC-3') + 1300R (5'-CACCAACTAAGAACGGCCATGC-3'). The amplification, PCR purification,

and sequencing were done as described for single-cell PCR. All strands were tested for consistency. The LSU rDNA amplification was achieved according to Carr et al. (2008).

Phylogenetic analysis

Alignments were carried out using Kalign (Lassmann and Sonnhammer 2005) and corrections were achieved manually. We calculated concatenated trees from 42 choanoflagellate taxa and about 4777 unambiguously aligned nucleotides (SSU and partial LSU rDNA) using maximum likelihood (ML) and Bayesian inference (BI) analysis. The Bayesian analysis was run with MrBayes v3.2.1 (Ronquist and Huelsenbeck 2003) using a GTR + I + Γ model and a four-category gamma distribution to correct for among site rate variation. MrBayes calculated the parameters for tree topology, branch length, nucleotide frequency, the individual nucleotide substitution rates, the proportion of invariable site, and the shape parameter of the gamma distribution for each partition. The analysis was performed for 1,000,000 generations with a "burnin" of 250 and an average standard split deviation <0.004452. The ML tree was performed by RAxML GUI 1.3 (Silvestro and Michalak 2012) using 1000 replicates and the GTRCAT model as suggested by the authors.

According to Nitsche et al. (2011), we used a two-taxa ichthyosporean clade (i.e. *Amoebidium parasiticum* SSU:Y19155/LSU:EU011932*, Ichthyophonus hoferi* SSU:U43712/LSU:AY026370) and a nine-taxa metazoan clade (i.e. *Beroe ovata* (SSU: AF293694/LSU:AF293694)*, Haliclona* sp. (SSU:KC902267/LSU:KC869594), *Hydra magnipapillata* (SSU:HQ392522/LSU:HQ392528)*, Leucosolenia* sp. (SSU:AF100945/LSU:AF100945), *Mnemiopsis leidyi* (SSU:AF293700 /LSU:AF293700)*, Nematostella vectensis* (SSU:AF254382/LSU:AY345871)*, Suberites domuncula* (SSU:AJ620112/LSU:AJ620112), *Sycon calcaravis* (SSU:D15066), *Trichoplax* sp. (SSU:Z22783/LSU:AY652581)) as outgroup. The following fifteen acanthoecid choanoflagellates were utilized for the tree computation: *Acanthoeca spectabilis* (SSU:AF084233/LSU:EU011933), *Acanthocorbis unguiculata*

(SSU:HQ026764), *Calliacantha* sp. (SSU:AF272000), Choanoflagellida sp. (SSU:HQ026765), *Diaphanoeca grandis* (SSU:L10824/LSU:EF681912), *Diaphanoeca pedicellata* (SSU:HQ237460), *Didymoeca costata* (SSU:EU011923/LSU:EU011938), *Helgoeca nana* (SSU:EF523335/LSU:EU011934), *Savillea micropora* (SSU:EU011928/LSU:EU011944), *Stephanoeca apheles* (SSU:EF523336), *Stephanoeca cauliculata* (SSU:HQ026766), *Stephanoeca diplocostata* (SSU:EU011927/LSU:EU011947), *Stephanoeca norrisii* (SSU:HQ026768), *Stephanoeca paucicostata* (SSU:HQ026769), uncultured eukaryote clone (SSU:JN090872).

The uncorrected pairwise distances between the SSU rDNA genes of the Craspedida were estimated using MEGA 6 (Tamura et al. 2013). All new sequences were deposited in GenBank (Table 1), alignments and pairwise distances are available from the author upon request.

Results

We isolated and (re-)described six species including one new genus. The descriptions of the genus/species are based on molecular (SSU and LSU rDNA) (Fig. 4) and morphological data. Theca morphology and the presence/absence of cyst formation and stalk are listed in Table 1. All morphological measurements are deposited as Supplementary Material (Supplement Table 1). The descriptions of the genus/species are top down arranged in order of their appearance in the phylogenetic tree. All new *“Salpingoeca”* species and the one *“Sphaeroeca”* species are set in quotation marks indicating that these species have to be renamed by future studies as this genus is not monophyletic (Fig. 4). To avoid any future renaming problems we strongly refrain from generating artificial genera until a large dataset on SSU sequences is available. Uncorrected pairwise distances between SSU rDNA genes of the Craspedida are deposited as supplementary data (Supplement Table 2).

Table 1. Locality, morphology and sequence information of the described species.

Species	Coordinates	Locality	Country	Habitat	Theca morphology	Cyst formation	Stalk	Accession number (NCBI) SSU rDNA	Accession number (NCBI) LSU rDNA
"Salpingoeca" ventriosa spec. nov.	69°5'N/ 49°48'W	SE of Ilulissat	Greenland	Freshwater	Flask-shaped	+	-	KJ631041	-
"Salpingoeca" longipes Saville Kent (1880)	39° 51'N/ 2° 47'E	SaCalobra, Mallorca	Spain	Marine	Cup (Glass)-shaped	+	+	KJ631040	KJ631046
"Sphaeroeca" leprechaunica spec. nov.	51° 47'N/ 9° 39'W	Gleninchaquin National Park, Kenmare	Republic of Ireland	Freshwater	No theca	+	n/a	KJ631042	KJ631047
"Salpingoeca" euryoecia spec. nov.	34° 52'S/ 56° 13'W	Arroyo Miguelete River	Uruguay	Estuary	Flask-shaped	+	+	KJ631038	KJ631045
"Salpingoeca" fusiformis Saville Kent (1880)	50° 49'N/ 1° 27'W	Beaulieu River	United Kingdom	Freshwater	Flask-shaped	+	+	KJ631039	KJ631044
Paramonosiga thecata gen. nov., spec nov.	50° 54'N/ 6° 58'E	River Rhine, Cologne	Germany	Freshwater	Flask-shaped	+	+	KJ631037	KJ631043

Abbreviations: +/- indicate whether the morphological character is apparent or not; n/a – not applicable.

Descriptions of one new genus and new and redescribed species

Order Craspedida Cavalier-Smith (1996)

Family Salpingoecidae

***"Salpingoeca" ventriosa* Jeuck, Arndt and Nitsche** spec. nov. Figs.: 1A-F, type strain: HFCC 1106.

Diagnosis: Freshwater *Salpingoeca*-like, about 8 x 6 µm in size with a flask-shaped and pot-bellied theca morphology, about 7.8 x 6.8 µm in size. A funnel-shaped theca; the anterior ending is deflecting to both ends. The collar has a width of about 1.7 µm with about 27 microvilli.

Etymology: *ventriosa* (adjective, feminine) from Latin "pot-bellied" in reference to the protruding posterior form of the theca.

Type locality: A freshwater lake in Greenland, 54 km South East of Ilulissat (69° 5'N/ 49° 48'E)

Holotype: The illustration of the specimen in Fig. 1A.

Description: *Salpingoeca*-like species with a body length of 7.5-8.6 µm (8.06 µm on average) and a body width of 4.2-7.5 µm (6 µm on average), long filopodia (see Sebé-Pedrós et al. 2013) occur (Fig. 1E). The theca has a pot-bellied protruding morphology at the posterior end. The anterior end consists of a funnel-shaped opening laterally deflecting to both ends (Figs. 1A-B, 1D). It is 6.9-8.87 µm in length (7.83 µm on average) and 6.4-7.3 µm in width (6.8 µm on average). The collar has a width of 1.4-2 µm (1.7 µm on average) at the base consisting of 23-30 (27 on average) relatively long microvilli. The base of the collar was observed to extend beyond the inner neck of the theca (Fig. 1F). The nucleus has a diameter of 2.2.-3.1 µm (2.7 µm on average) and the nucleolus is 0.6-1.4 µm (on average 1.1 µm) in diameter. Food particles (with an average size of 0.8 µm) are transported along the microvilli (tentacles) via a feeding pseudopodium (Fig. 1F). Cyst formation with a thick cyst wall occurs (Fig. 1C).

Type sequence data: The SSU rDNA sequence data of *"S." ventriosa* spec. nov. has been deposited in the GenBank database with the accession number as follows: SSU: KJ631041. The closest relative sequence on NCBI nucleotide BLAST® results in an uncultured eukaryote freshwater clone (JN090879) with a maximum identity of 93%.

Remarks: Species with slightly similar theca morphology, but a two layered theca are *Diploeca angulosa* De Saedeleer (1927) and *Diploeca flava* (Korshikov 1926) Bourrelly (1957). The theca of *S. ventriosa* has a funnel-shaped opening. In addition, it is more protruding at both ends (pot-bellied form) than *Diploeca* species.

"Salpingoeca" longipes Saville Kent (1880) sensu Boucaud-Camou (1967) and Tong (1997); Figs.: 1G-L, neotype strain: HFCC 1114.

Remarks: Marine *Salpingoeca*-like species, found in the bay of Sa Calobra (Mallorca, Spain) with cup-shaped theca morphology. Neotypified (1) as no type material is available, (2) the original morphological description lacks many morphological features making an unquestionable assignment difficult, and (3) to relate a well-defined genotype to a well-defined morphotype. Additional morphological measurements and first sequence data are presented to improve the diagnosis.

Emended diagnosis: The species has a body length of 3.1-7.8 µm (4.6 µm on average) and a body width of 2.7-4.4 µm (3.4 µm on average). The collar length can be very variable and may range from 1.6 to 9.8 µm (6.1 µm on average). The collar width can be 1.5-3.7 µm (2.2 µm on average) at its base and ranges from 2 to 7.5 µm (5.3 µm on average) at its top. The cell body occupies approximately half to one third of the glass-shaped theca cavity (Fig. 1J). The theca has a length of 4.1-9.5 µm (6.9 µm on average) and a width of 2.1-5 µm (3.5 µm on average). It shows a thickened posterior end at the transition to the stalk (Fig. 1J). The stalk is relatively long with 15.6-43.9 µm (36.5 µm on average) (Fig. 1L). The diameter of the centrally to posteriorly located nucleus is 1.4-3 µm (1.8 µm on average), whereas the nucleolus is 0.8-1.5 µm (1 µm on average) in diameter. The flagellum varies from 7.6-16.6 µm in length (13.2 µm on average) and the vacuoles have an average diameter of 0.8 µm.

Cyst formation with a thick cyst wall occurs (Fig. 1H). Fast swimmers with a long flagellum (Fig. 1K) and with a short flagellum contacting the microvilli of a thecate cell (Fig. 1I) have been observed.

Neotype sequence data: The SSU rDNA sequence data and parts of the LSU rDNA of *"Salpingoeca" longipes* have been deposited in the GenBank database with the accession numbers as follows: SSU: KJ631040, LSU: KJ631046. The closest relative sequence of the SSU rDNA on NCBI nucleotide BLAST® results in "*Salpingoeca" infusionum* (AF100941) with a maximum identity of 98%.

Remarks: Saville Kent (1880) describes a stalk of great length (4-5 times the length of the cell body) as a distinct feature of recognition and a cell body occupying two-thirds of the cavity of the theca (Fig. 1G). In addition, Ruinen (1938) indicates a cell size of 6x3 µm and a theca size of 9x3 µm. All these measurements correspond to our observations. Despite, the gene sequence data indicate a very close relationship to *"Salpingoeca" infusionum* confirmed by the very similar morphology. Boucaud-Camou (1967) uses *"Salpingoeca" infusionum* and *"Salpingoeca" longipes* as synonyms explaining that different stalk lengths occur. In addition, Tong (1997) describes a species of *"Salpingoeca" infusionum* with a very long stalk (5-7 times as long as the cell, 11.5-27 µm), a theca length of 2-2.5 times the width, and a "bulb" at the base of the theca. Since this corresponds to our observations of *"Salpingoeca" longipes*, we argue that Boucaud-Camou's and Tong's description of *"Salpingoeca" infusionum* with a long stalk correspond to *"Salpingoeca" longipes* rather than to *"Salpingoeca" infusionum*. In addition, the only 98% identity with *"Salpingoeca" infusionum* (p-distance: 1.5%) is representing a proxy for organisms belonging to the same genus rather than the same species (Bachy et al. 2013).

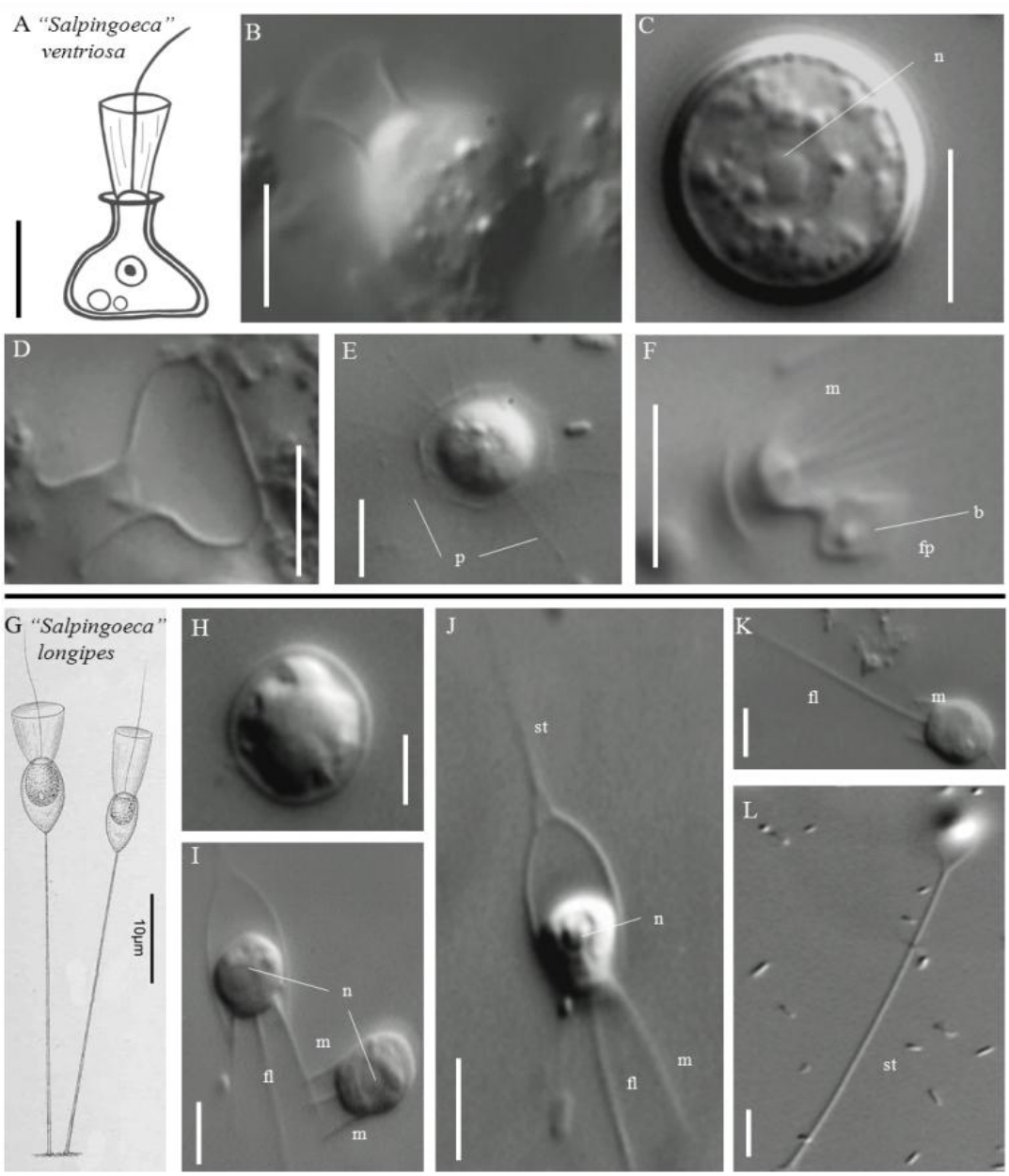

Figure 1. General morphology of *"Salpingoeca" ventriosa* and "*Salpingoeca" longipes.* **A-F:** *"Salpingoeca" ventriosa*. **A:** Schematic illustration of the specimen; **B:** Theca morphology with cell body; **C:** Cyst; **D:** Empty theca; **E:** Cell body forming pseudopodia; **F:** Collar with microvilli and feeding pseudopodium; **G-L:** *"Salpingoeca" longipes*. **G:** Illustration of the specimen by Saville Kent (1880); **H:** Cyst; **I:** Thecate specimen and fast swimming specimen with short collar; **J:** Thecate specimen with nucleus; **K:** Fast swimmer with long flagellum; **L:** Stalk. Scalebar: 5µm, if not indicated. Abbreviations: b – bacterium; fl – flagellum; m - microvilli; n - nucleus; p - pseudopodium; st – stalk.

***"Sphaeroeca" leprechaunica* Jeuck, Arndt and Nitsche** spec. nov. Figs.: 1A-D, type strain: HFCC 1104.

Diagnosis: Freshwater *Sphaeroeca*-like species, about 5.7 x 4.4 µm in size with a flagellum, about 10 µm long and a collar, about 6.2 x 2.2 µm in size. Short filopodia may occur posteriorly. The ability to form colonies has not been observed up to now.

Etymology: *leprechaunica* (adjective, feminine), latinized, in reference to the Irish fairy "Leprechaun", indicating that the species was isolated in the Republic of Ireland.

Type locality: A freshwater stream in Gleninchaquin National Park (51° 47'N/9° 39'W), south-western part of the Republic of Ireland with a salinity of about 0 PSU.

Holotype: The illustration of the specimen in Fig. 2A.

Description: *Sphaeroeca*-like species with a body length of 3.9-7.1 µm (5.7 µm on average) and a body width of 2.9-5.8 µm (4.4 µm on average). The flagellum is 7.5-11.5 µm long (9.8 µm on average). The collar has a length of 6-6.3 µm (6.2 µm on average). The collar width is 1.2-3 µm (2.2 µm on average) at its basis and 5.1-6.9 µm (5.9 µm on average) at its top (Fig. 2D). 2-3 vacuoles (contractile and feeding) are located at the posterior end with an average diameter of 1.7 µm (Figs. 2C, 2D). Short filose pseudopodia are present at the posterior end (Figs. 2C-D). No colony formation and no stalk have been observed. The middle to anterior positioned nucleus has a diameter of 1.4-3.1 µm (2.2 µm on average) and the nucleolus is 0.6-2 µm (on average 1.2 µm) in diameter (Figs. 2B, 2D). Food particles (with an average size of 1 µm) are ingested via a pocket-like feeding pseudopodium (as observed for *Codosiga*, compare Stoupin et al. 2012). Cyst formation occurs.

Type sequence data: The SSU rDNA sequence data and parts of the LSU rDNA of *"Sphaeroeca" leprechaunica* spec. nov. have been deposited in the GenBank database with the accession numbers as follows: SSU: KJ631042, LSU: KJ631047. The closest relative sequence of the SSU rDNA on NCBI nucleotide BLAST® results in *Sphaeroeca volvox* (Z34900) with a maximum identity of 94%.

Remarks: Up to now, no colonies have been observed in *"Sphaeroeca" leprechaunica* in contrast to the other representatives of the genus *Sphaeroeca*. In addition, it differs from all other *Sphaeroeca* species *(Sphaeroeca volvox* Lauterborn 1894, *Sphaeroeca pedicellata* (Oxley) Lemmermann (1910), *Sphaeroeca globosa* Wawrik (1956), *Sphaeroeca salina* Bourrelly (1957), *Sphaeroeca lackey* Bourrelly (1968), *Sphaeroeca desmarelloides* Wawrik (1981) in the absence of a stalk and in the length of the flagellum. Lauterborn (1894) indicates a flagellum of *Sphaeroeca volvox*, which is five times as long as the cell body (cell body: ca. 8 µm), whereas the flagellum length of *Sphaeroeca pedicellata* is 2-2 ½ as long as the cell body (cell body: 10-13.6 µm, Skuja 1932); Wawrik (1956) observes a flagellar length of *Sphaeroeca globosa* of 55-70 µm. According to Bourrelly (1957), *Sphaeroeca salina* has a flagellum length of 20-25 µm, whereas *Sphaeroeca lackey* has a flagellum length of 50 µm (Zhukov 1993), and Wawrik (1981) indicates a flagellum length of *Sphaeroeca desmarelloides* of 20 µm.

"*Sphaeroeca" leprechaunica* is clustering with *"Sphaeroeca" volvox* with a genetic p-distance of 4.5%. No morphological descriptions were added to the sequence data of *"Sphaeroeca" volvox* available in GenBank (Z34900) and we have not been able to observe colonial stages in *"Sphaeroeca" leprechaunica*. Thus we cannot confirm possible colonial stages in the life cycle.

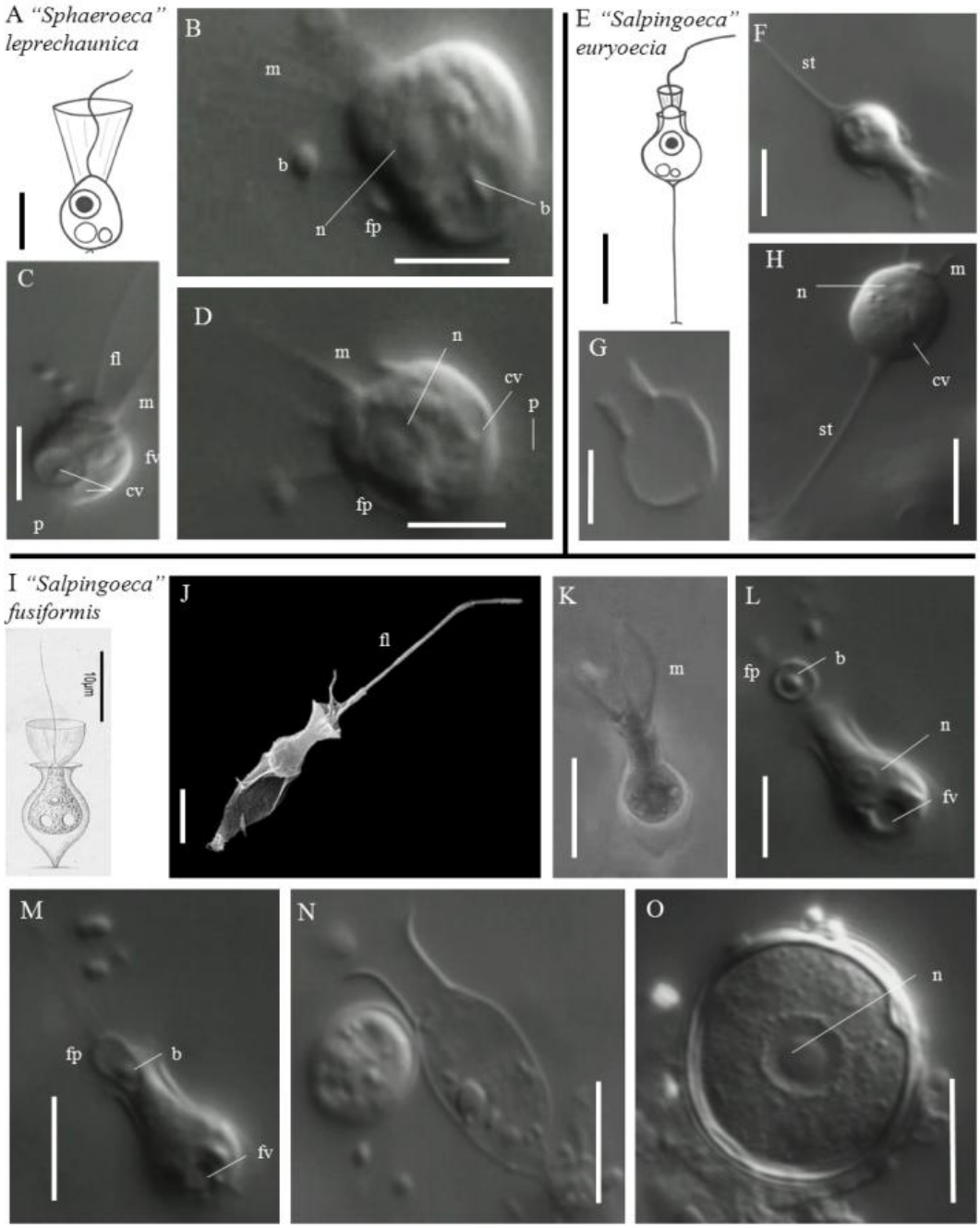

Figure 2. General morphology of *"Sphaeroeca" leprechaunica, "Salpingoeca" euryoecia*, and *"Salpingoeca" fusiformis.* A-D: *"Sphaeroeca" leprechaunica*. **A:** Schematic illustration of the specimen; **B-D:** Fine structure of the cell; **E-H:** *"Salpingoeca" euryoecia*. **E:** Schematic illustration of the specimen; **F:** Thecate specimen with stalk; **G:** Empty theca **H:** Fine structure of a non-thecate cell; **I-O:** *"Salpingoeca" fusiformis*. **I:** Illustration of the specimen by Saville Kent (1880); **J:** Electron micrograph of a thecate cell; **K:** Phase contrast

micrograph of a thecate cell; **L:** Fine structure of the cell and feeding pseudopodium; **M:** Fine structure of the cell and feeding pseudopodium; **N:** Empty theca; **O:** Cyst. Scalebar: 5µm, if not indicated. Abbreviations: b – bacterium; cv – contractile vacuole; fl – flagellum; fp – feeding pseudopodium; fv – feeding vacuole; m - microvilli; n - nucleus; p - pseudopodium; st – stalk.

***"Salpingoeca" euryoecia* Jeuck, Arndt and Nitsche** spec. nov. Figs.: 2E-H; type strain: HFCC 1100.

Diagnosis: Freshwater *Salpingoeca*-like species, about 4.9 x 3.7 µm in size with a collar, about 1.3 µm long. The flask-shaped theca is about 4.6 µm long and 2.5 µm wide. A stalk is present with a length of about 9.4 µm.

Etymology: *euryoecia* (adjective, feminine), latinized, in reference to the fact that this species is euryoecious, because it is able to tolerate salinities ranging from fresh to brackish water level.

Type locality: Arroyo Miguelete River Estuary in Montevideo, Uruguay (34° 52'S/ 56° 13'W), with a salinity of about 0 PSU.

Holotype: The illustration of the specimen in Fig. 2E.

Description: Thecate craspedid choanoflagellate species with an oval, elongated cell body, 3.6-6.1 µm in length (4.9 µm on average) and 1.3-4.7 µm in width (3.7 µm on average). The flask-shaped theca has a length of 5.3-5.9 µm (4.61 µm on average) and a width of 3.4-4.3 µm (2.5 µm on average) (Figs. 2E-G). The collar has a length of 0.8-1.6 µm (1.3 µm on average) with a width of 0.9-1.6 µm at its base (1.3 µm on average) and 0.7-1.8 µm at its top (1.5 µm on average). 2 vacuoles (contractile and feeding) with an average diameter of 0.9 µm are located at the posterior end (Fig. 2H). The position of the nucleus is central to anterior, it has a diameter of 1.3-2 µm (1.6 µm on average) and the nucleolus is 0.4-0.7 µm (0.6 µm on average) in diameter. The length of the stalk is 7-10.8 µm (9.4 µm on average). Many stalked cells occur without a theca (Fig. 2H).

Type sequence data: The SSU rDNA sequence data and parts of the LSU rDNA of *“Salpingoeca” euryoecia* have been deposited in the GenBank database with the accession numbers as follows: SSU: KJ631038, LSU: KJ631045. The closest relative sequence of the SSU rDNA on NCBI nucleotide BLAST® results in an uncultured freshwater eukaryote clone (GU290096) with a maximum identity of 90%.

Remarks: This craspedid species differs from all other thecate craspedid species by its specific theca morphology and the relatively short collar. Ellis (1930) described a brackish water *Salpingoeca de-saedeleeri* with cell body and theca measurements (height of lorica body: 6µm; length of lorica neck: 2.5µm; extreme width of lorica: 8µm; diameter of lorica mouth: 4µm; length of cell (contracted): 5µm; width of cell: 4µm) corresponding to *“Salpingoeca” euryoecia*, but without any measurements of the stalk, just described as “short”. In addition, the collar of *Salpingoeca de-saedeleeri* is described as “long” (no measurements) and the theca has a squat morphology; both characteristics are significantly different to *“Salpingoeca” euryoecia*. Other similar species are *Salpingoeca cardiforma* Ellis (1930), *Salpingoeca huxleyi* Ellis (1930) and *Salpingoeca stenotheca* Boucaud-Camou (1967), but all differ significantly in the specific measurements of dimensions and the theca morphology.
“Salpingoeca” euryoecia is clustering with *Desmarella moniliformis*, but showing a high genetic p-distance of 11.3%. Both species possess a flask-shaped theca (Fig. 2E and Leadbeater and Karpov 2000), but no chain-like (ribbon-shaped) colony, as described for *Desmarella moniliformis,* was observed for *“Salpingoeca” euryoecia.*

"Salpingoeca" fusiformis Saville Kent (1880); Figs.: 2I-O, neotype strain: HFCC 1101.

Remarks: Freshwater *Salpingoeca*-like species, isolated from River Beaulieu (South-England) with flask-shaped theca morphology very similar to the original description by Saville Kent (1880-82). Some additional morphological measurements, first sequence data, and electron microscopical image are supplied. Hence, an improved diagnosis is here provided based on previous and present studies.

Improved diagnosis: The ovoid to elongated freshwater species has a body length of 3.8-9.7 µm (6.2 µm on average) and a body width of 2.8-4.4 µm (3.8 µm on average). It possesses a flask-shaped theca with a length of 4.3-12.1 µm (7.2 µm on average) and a width of 2.3-4.7 µm (3.9 µm on average). The anterior end of the theca neck is everted to both sides (Figs. 2I-J, 2N). The collar is 3.8-7.6 µm long (5.7 µm on average), 0.9-1.8 µm (1.4 µm on average) wide at its basis and 3.7-6 µm (4.9 µm on average) at its top. It is consisting of 20-24 microvilli. The diameter of the centrally located nucleus is 1.7-2.8 µm (2.1 µm on average), whereas the nucleolus is 0.7-1.2 µm (0.9 µm on average) in diameter. 2 vacuoles, with an average diameter of about 1.2 µm, are located at the posterior end (Figs. 2L-M). Cyst formation with a thick cyst wall occurs (Fig. 2O). Food particles (with an average size of 0.7 µm) are ingested via phagocytosis with a feeding pseudopodium (Figs. 2L-M).This corresponds to Saville Kent (1880-82) describing an irregular lobate process of the exuding cell body.

Neotype sequence data: The SSU rDNA sequence data and parts of the LSU rDNA of *"Salpingoeca" fusiformis* have been deposited in the GenBank database with the accession numbers as follows: SSU: KJ631039, LSU: KJ631044. The closest relative sequence of the SSU rDNA on NCBI nucleotide BLAST® results in *Monosiga ovata* (AF271999) with a maximum identity of 92%.

Genus *Paramonosiga* Jeuck, Arndt and Nitsche gen. nov.

Diagnosis: Small, ovoid to anteriorly truncated cells, with one flagellum, centrally and anteriorly located. Flagellum surrounded by a collar consisting of microvilli. Different life cycle stages may occur. When a theca is present, then with a flask-shaped morphology. The theca is ending into the thick stalk. Phagotrophic filter feeders. Freshwater. Distinct from all other genera of Craspedida due to its separate phylogenetic position. Type species: *Paramonosiga thecata* Jeuck et al.

Etymology: *Paramonosiga*, from ancient-greek “para”, because not being *Monosiga*, but its phylogeny and morphology is “next to” *Monosiga.*

***Paramonosiga thecata* Jeuck, Arndt and Nitsche** spec. nov. Figs.: 3A-H; type strain: HFCC 45.

Diagnosis: Freshwater *Paramonosiga*, about 6.2 x 5.2 µm in size with a flagellum, about 8.5 µm long and a collar with a size of about 5.7 x 3.5 µm. Under unknown conditions, a flask-shaped theca is produced with a size of about 8 x 3.7 µm and a stalk, about 3 µm long.

Etymology: *thecata* (adjective, feminine) latinized, in reference to the fact that a theca morphology is built from time to time.

Type locality: The River Rhine at Cologne, Germany (50° 54’N/6° 58’E).

Holotype: The illustration of the specimen in Fig. 3A/4C.

Description: *Paramonosiga* species with an ovoid, anteriorly truncated cell body, 4.6-8.1 µm in length (6.2 µm on average) and 3.9-6.2 µm in width (5.2 µm on average). Flask-shaped theca morphology with a length of 6-10 µm (8 µm on average) and a width of 3.2-5.2 µm (3.7 µm on average) occurs under certain not yet analysed conditions and within irregular time intervals (compare Dayel et al. 2011 for *“Salpingoeca” rosetta*). Both ends of the theca are posteriorly and equally tapering towards the thick stalk (Fig. 3C). The length of the thickened theca stalk is 1.6-4.6 µm (3 µm on average) (Fig. 3C, H). The flagellum is 6.9-9.8 µm long (8.5 µm on average)

(Fig. 3D, G) and the collar has a length of 3.8-8.6 µm (5.7 µm on average) consisting of 20-24 microvilli (Fig. 3D). The collar width is 2.5-4.4 µm at its base (3.5 µm on average) and 5.2-10.4 µm at its top (8 µm on average). 2 vacuoles (contractile and feeding) with an average diameter of about 2.2 µm are located at the posterior end (Fig. 3E). The nucleus is centrally to anteriorly positioned, it has a diameter of 1.2-3.2 µm (2.5 µm on average) and the nucleolus is 0.8-2.5 µm (1.7 µm on average) in diameter (Figs. 3E-H). Longest food particles ingested had an average size of 1.2 µm (maximum: 2.5 µm). Fast swimming forms with a short collar exist (Fig. 3B, G).

Type sequence data: The SSU rDNA sequence data and parts of the LSU rDNA of *Paramonosiga thecata* have been deposited in the GenBank database with the accession numbers as follows: SSU: KJ631037, LSU: KJ631043. The closest relative sequence of the SSU rDNA on NCBI nucleotide BLAST® results in an uncultured freshwater eukaryote clone (GU647194) with a maximum identity of 89%.

Remarks: The *Paramonosiga* species is morphologically indistinguishable from other monosigid species as it also possesses an ovoid cell body (e.g. *Monosiga brevipes* Saville Kent 1880; *Monosiga fusiformis* Saville Kent 1880; *Monosiga gracilis* Saville Kent 1880; *Monosiga gemina* De Saedeleer 1927).

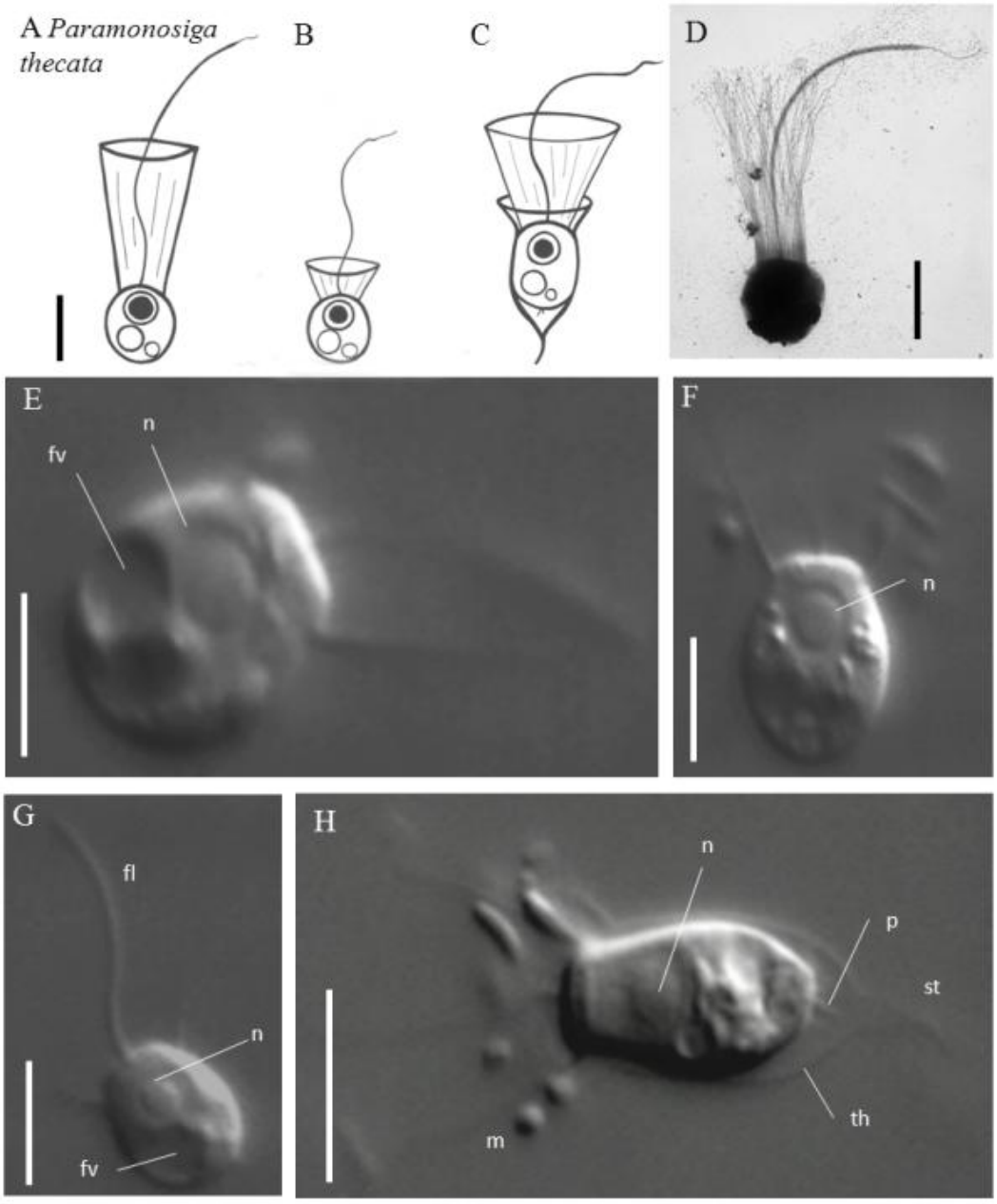

Figure 3. General morphology of *Paramonosiga thecata.* **A:** Schematic illustration of the non-thecate specimen; **B:** Schematic illustration of the fast swimming specimen; **C:** Schematic illustration of the thecate specimen; **D:** Electron micrograph of the thecate cell; **E-F:** Fine structure of the non-thecate cell; **G:** Fast swimmer with flagellum; **H:** Fine structure of the thecate cell. Scalebar: 5µm. Abbreviations: fl – flagellum; fv – feeding vacuole; m - microvilli; n - nucleus; p - pseudopodium; st – stalk; th - theca.

Phylogenetic analysis

The partial SSU and LSU rDNA of the six species were sequenced (Table 1). The concatenated phylogeny (Fig. 4) extended by the novel results of the present study generally confirms the topology of previous studies (Carr et al. 2008; Nitsche et al. 2011; Wylezich et al. 2012). Some new relationships and a new genus are uncovered due to the addition of sequence data.

The 42 taxa of choanoflagellates and 9 taxa of Metazoa form a distinct branch with strong support (1.00 biPP [Bayesian interference posterior probability] and 100% mlBP [maximum likelihood bootstrap percentage]). We have strong evidence for the monophyly of the Craspedida (0.99 biPP; 99% mlBP) and for the Acanthoecida (0.99 biPP; 100% mlBP). Clade 1 is in accordance to clade 1 in Carr et al. (2008) with the addition of the *Codosiga* species from Wylezich et al. (2012) and our isolates from Greenland, *"Salpingoeca" ventriosa,* and Spain, *"Salpingoeca" longipes.* All branches are highly bootstrap supported by biPP, and high to moderate mlBP.

The isolate *"Salpingoeca" ventriosa* clusters separately within the stable Craspedida clade 1; this clade is a sister to the clade consisting of *"Salpingoeca" infusionum* and *"Salpingoeca" longipes* with strong support (1.00 biPP; 100% mlBP). *"Salpingoeca" longipes* is very closely related to *"Salpingoeca" infusionum* (p-distance: 1.5%, Supplement Table 2) which is in agreement with similarities in their morphology. *"Sphaeroeca" leprechaunica* is forming a clade with *Sphaereoca volvox* (1.00 biPP; 100% mlBP).

Clade 2 in the present study corresponds to Clade 2 in Carr et al. (2008): *"Salpingoeca" euryoecia*, *"Salpingoeca" fusiformis* and *Paramonosiga thecata* are all clustering within clade 2, but the added sequences have revealed new branching relationships. The isolate from Uruguay, *"Salpingoeca" euryoecia*, is related to *Desmarella moniliformis* (1.00 biPP; 63% mlBP) and we assigned it to *"Salpingoeca"* according to its thecate morphology. *Desmarella moniliformis* may also show flask-shaped theca forms (see Leadbeater and Karpov 2000). The species from the UK, which we redescribe as *"Salpingoeca" fusiformis*, forms a separate branch but with

relatively low bootstrap support: 0.91 biPP; <50% mlBP. The new species from Germany is deeply and separately branching with moderate support (0.94 biPP; 61% mlBP). For this reason we have erected the new genus *Paramonosiga* and named this species *Paramonosiga thecata.*

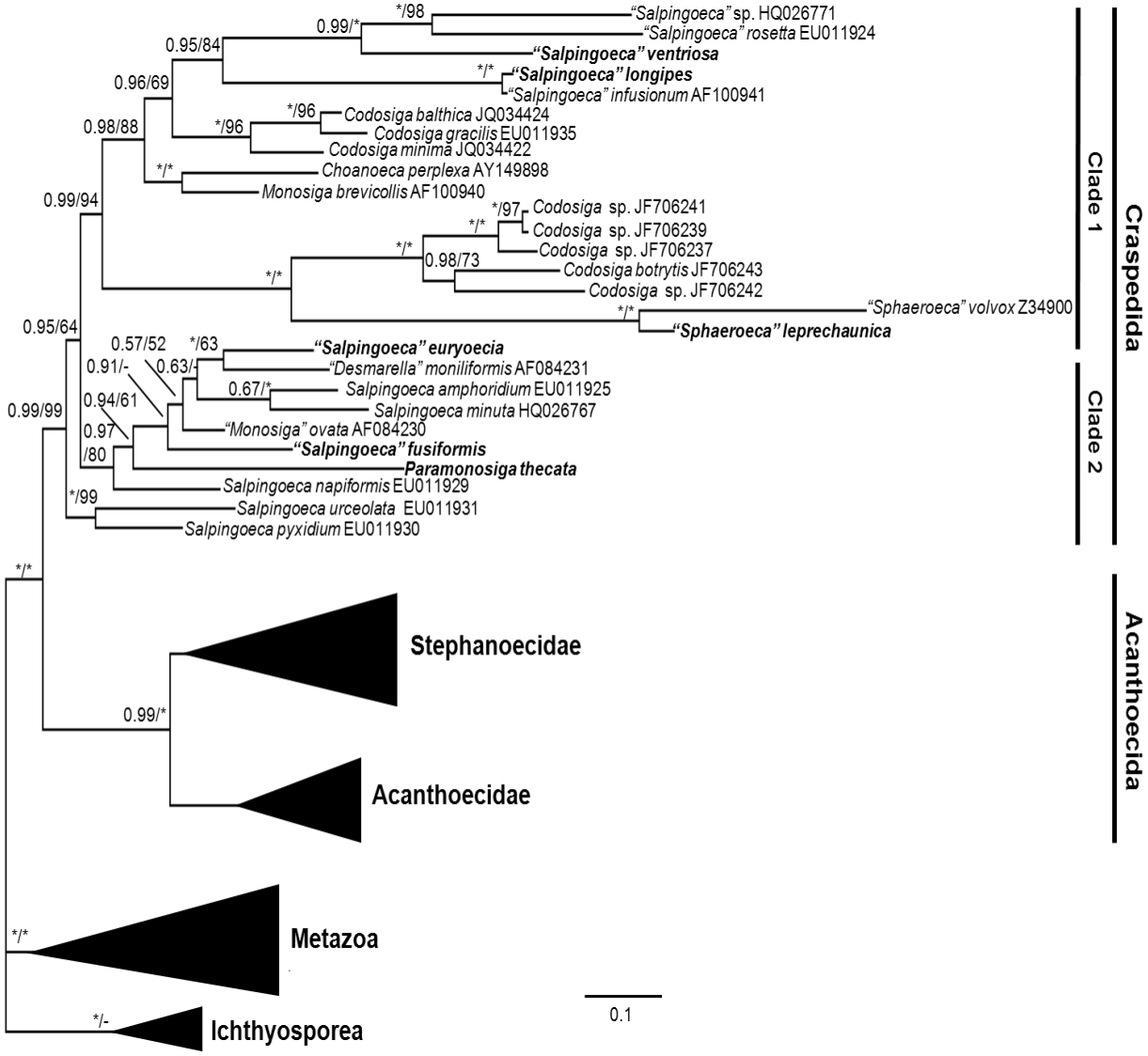

Figure 4. Concatenated maximum likelihood phylogeny of the choanoflagellate SSU and LSU rDNA sequences (4777 nt). The six newly sequenced species are marked by bold letters. Support values are offered for BI/RAxML at each node. 1.00 BI posterior probabilities (biPP) and 100 % RAxML bootstrap percentage support (mlBP) are denoted by *. Support values under 50% mlBP are indicated by a -. The scale bar in the lower middle indicates the

number of nucleotide substitutions per site. Accession numbers of the newly described species are listed in Table 1.

Discussion

We have been able to extend the phylogeny of the order Craspedida by adding six new sequences and morphological descriptions. Based on these rDNA sequences, we erected one new genus and assigned two sequences to formerly described *Salpingoeca* species. The four other species have been described as new species. The species of *"Salpingoeca"*, clade 1, i.e. *"Salpingoeca" infusionum* and *"Salpingoeca" longipes,* (Fig. 4) are characterised by their very close relationship to each other. Based on the genetic distance, we have therefore rejected the hypothesis of Boucaud-Camou (1967) synonymising *"S." longipes* as *"S." infusionum*.

The new genus *Paramonosiga* was erected because of its phylogenetic position within the Craspedida. Although morphologically undistinguishable from the *Monosiga*-like forms, the high p-distance of 14.5% supports this erection of a new genus. Stokes (1883) described *Monosiga woodiae* as a thecate *Monosiga* species. Unfortunately, he did not offer theca measurements; additionally, his drawing shows a different theca morphology (narrowed, theca ends not as tapering as within *Paramonosiga thecata*). Since Dayel et al. (2011) detected different life cycle forms attributable to *"Salpingoeca" rosetta*, we hypothesis that *Paramonosiga thecata* could also possess a complex life cycle, especially due to the fact that also fast swimming forms with a short collar exist (Fig. 3B, 3G).

Though we have extended the present available dataset on described choanoflagellates (NCBI) by about one fifth, we refrain from altering the present taxonomy as this will only lead to future renaming issues. To avoid this problem, a large number of additional species sequences including morphological descriptions must be obtained. As the genus *Salpingoeca* is clearly paraphyletic in its present form, we acknowledge its need of revision by using quotation marks in this study. A major hindrance to undertaking a re-appraisal is that the type specimen *Salpingoeca gracilis* (described by James-Clark 1867) has not yet been sequenced and hence a

clear assignment of the genus *Salpingoeca* is not possible. One might speculate that this species, as it is described from freshwater, might likely cluster within clade 2. But based on the data from this study no clustering due to ecological parameters like salinity of the habitat could be detected. The same is applicable for the assumption that *S. gracilis* forms a new clade of tube-shaped species as showing this kind of theca morphology.

The major problem within choanoflagellate taxonomy and systematics as currently conceived is the fact that a genus distinguishing feature only occurs in certain stages of the life cycle. As mentioned above, Dayel et al. (2011) detected different life cycle forms of *"Salpingoeca" rosetta* comprising *Monosiga*-like, *Proterospongia*-like, *Desmarella*-like, and *Codosiga*–like forms. The designation of these forms to either life cycle forms or genera is a task for future studies. It is very likely that four of the six new species clustering within clade 1 (*"Salpingoeca" ventriosa* and *"Salpingoeca" longipes*) and clade 2 (*"Salpingoeca" euryoecia* and *"Salpingoeca" fusiformis*) will have to be renamed in further studies, based on even more sequence data. In addition, *"Salpingoeca" euryoecia* and *"Desmarella" moniliformis* forming one group within clade 2, would have to be assigned to a new genus when obtaining more certain bootstrap support. Unfortunately no morphological information relating to other life cycle stages of *D. moniliformis* is currently available, indicating that again the problem of life cycle dependant variability might have led to a misidentification. Besides, both are capable of forming flask-shaped coverings (Leadbeater and Karpov 2000). Furthermore, *"Salpingoeca" fusiformis,* clustering with *"Monosiga" ovata*, will also have to be renamed, since the genus *Monosiga* is paraphyletic.

We could not observe colony formation within our culture of *"Sphaeroeca" leprechaunica* as this typical colony formation was described as the distinct morphological feature of recognition of *Sphaeroeca* species. Thus we refrain from establishing a new genus for *"Sphaeroeca" leprechaunica* as this might cause further confusion in choanoflagellate taxonomy. Nevertheless, we set the genus name *"Sphaeroeca"* in quotation marks to indicate that the sequence data revealed a close relationship to *"Sphaeroeca" volvox* on the one hand, but renaming has to be postponed on the other hand when more sequence data will be available. In addition,

no morphological descriptions were added to the isolate of *“Sphaeroeca” volvox* published as sequence data (Z43900) and therefore it is questionable whether this isolate was able to form colonial stages at all. The *“Sphaeroeca”* species are clustering within the *Codosiga botrytis* morphospecies complex (compare Stoupin et al. 2012). This is remarkable regarding morphological aspects. Both genera are quite similar comparing the general form of the cell body. In addition, *“Sphaeroeca” leprechaunica* and the *Codosiga botrytis* morphospecies complex are both forming similar feeding pseudopodia laterally on the cell body (see Fig. 2B, 2D for *“Sphaeroeca” leprechaunica*; see Stoupin et al. (2012) for *Codosiga botrytis* complex).

Apart from that, this study revealed that it is currently not possible to use the theca morphology (flask-, cup-, tube-shaped) as a phylogenetic feature of recognition. The habitat preference of the species (marine, freshwater) may also not be used as a phylogenetic feature as the results are unambiguous: No correlation of morphology and/or ecology could be detected in the phylogenetic analysis.

To conclude, we hope that this extended set of morphological descriptions and sequences will help to classify and reorder craspedid choanoflagellates in future.

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Supplementary Material

Table 2. Morphometric data of the described species

Species	Character	Mean	Min	Max	Median	SD	SE	CV %	n
"Salpingoeca" ventriosa spec. nov.	Body length	8.06	7.5	8.6	8.06	0.47	0.2	0.4	4
	Body width	6.0	4.2	7.5	6.0	0.87	0.12	0.77	49
	Theca length	7.83	6.9	8.87	7.72	0.83	0.3	0.73	7
	Theca width	6.78	6.45	7.26	6.80	0.22	0.05	22	0.19
	Collar width at basis	1.7	1.4	2.0	1.7	0.28	0.08	0.25	13
	Microvilli number	27	23	30	27	2.55	1.02	2.26	5
	Diameter nucleus	2.7	2.2	3.1	2.8	0.29	0.07	0.26	15
	Diameter nucleolus	1.1	0.6	1.4	1.2	0.23	0.06	0.20	15
	Flagellum length	10.0	10.0	10.0	10.0	0.00	0.00	0.00	1
	Diameter vacuole	1.5	0.7	2.9	1.4	0.55	0.08	0.49	49
	Longest food particle ingested	0.85	0.76	1.04	0.81	0.16	0.05	0.14	7
"Salpingoeca" longipes Saville Kent (1880)	Body length	4.6	3.1	7.8	4.3	1.41	0.33	30.33	17
	Body width	3.4	2.7	4.4	3.6	0.54	0.12	15.80	21
	Theca length	6.88	4.15	9.45	6.91	1.97	0.49	28.56	15
	Theca width	3.47	2.1	4.95	3.06	0.87	0.21	25.04	16
	Collar length	6.1	1.6	9.8	6.0	2.46	0.68	40.12	12
	Collar width at basis	2.2	1.5	3.7	2.1	0.52	0.13	23.61	16
	Collar width at top	5.3	2.0	7.5	5.8	1.65	0.46	31.39	12
	Diameter nucleus	1.8	1.4	3.0	1.7	0.55	0.19	30.17	7
	Diameter nucleolus	1.0	0.8	1.5	0.9	0.28	0.11	27.21	6
	Flagellum length	13.2	7.6	16.6	14.4	4.15	1.80	31.34	4
	Diameter vacuole	0.8	0.7	0.9	0.8	0.16	0.08	20.20	2
	Stalk length	36.5	15.6	43.9	42.6	11.44	4.26	31.33	6
"Sphaeroeca" leprechaunica spec. nov.	Body length	5.7	3.9	7.1	5.9	0.90	0.16	15.63	32
	Body width	4.4	2.9	5.8	4.4	0.61	0.10	14.12	39
	Collar length	6.2	6.0	6.3	6.2	0.25	0.12	4.01	2
	Collar width at basis	2.2	1.2	3.0	2.1	0.50	0.11	22.92	21
	Collar width at top	5.9	5.1	6.9	5.8	0.67	0.27	11.30	5

	Microvilli number	30	30	30	30	0	0	0	1
	Diameter nucleus	2.2	1.4	3.1	2.3	0.43	0.10	19.44	19
	Diameter nucleolus	1.2	0.6	2.0	1.2	0.30	0.07	26.06	18
	Flagellum	9.8	7.5	11.5	10.1	1.70	0.73	17.31	4
	Diameter vacuole	1.7	0.9	2.9	1.7	0.39	0.05	22.03	58
	Longest food particle ingested	1.0	0.7	1.2	1.0	0.16	0.05	16.25	9
"Salpingoeca" euryoecia spec. nov.	Body length	4.9	3.6	6.1	4.8	0.50	0.08	10.40	42
	Body width	3.7	1.3	4.7	3.7	0.42	0.05	11.41	74
	Theca length	4.61	5.34	5.88	5.24	0.48	0.18	9.06	6
	Theca width	2.53	3.43	4.26	3.46	0.60	0.20	17.56	8
	Collar length	1.3	0.8	1.6	1.3	0.21	0.05	16.30	19
	Collar width at basis	1.3	0.9	1.6	1.3	0.19	0.04	14.81	21
	Collar width at top	1.5	0.7	1.8	1.5	0.31	0.07	20.75	19
	Diameter nucleus	1.6	1.3	2.0	1.6	0.15	0.02	9.22	45
	Diameter nucleolus	0.6	0.4	0.7	0.6	0.10	0.01	17.75	47
	Diameter vacuole	0.9	0.6	1.3	0.9	0.15	0.02	16.96	45
	Stalk length	9.4	7.0	10.8	10.1	1.47	0.46	15.72	9
"Salpingoeca" fusiformis Saville Kent (1880)	Body length	6.2	3.8	9.7	6.0	1.40	0.27	22.79	26
	Body width	3.8	2.8	4.4	3.9	0.40	0.06	10.47	34
	Theca length	7.24	4.26	12.1	6.97	1.86	0.39	25.67	22
	Theca width	3.92	2.30	4.72	3.92	0.41	0.07	10.52	30
	Collar length	5.7	3.8	7.6	5.8	1.52	0.53	26.48	7
	Collar width at basis	1.4	0.9	1.8	1.4	0.23	0.04	16.07	26
	Collar width at top	4.9	3.7	6.0	5.0	0.78	0.27	15.78	7
	Microvilli number	22	20	24	22	2.83	1.41	12.86	2
	Diameter nucleus	2.1	1.7	2.8	2.0	0.32	0.07	15.27	22
	Diameter nucleolus	0.9	0.7	1.2	0.9	0.13	0.03	15.31	21
	Diameter vacuole	1.2	0.7	2.1	1.2	0.28	0.04	23.74	48
	Longest food particle ingested	0.7	0.7	0.7	0.7	0	0	0	1
Paramonosiga thecata gen. nov., spec. nov.	Body length	6.2	4.6	8.1	6.1	0.85	0.12	13.92	53
	Body width	5.2	3.9	6.2	5.2	0.67	0.09	12.85	52

Theca length	8.0	6.0	10.02	8.18	1.17	0.29	14.25	15
Theca width	3.75	3.23	5.18	3.69	0.5	0.13	13.68	15
Collar length	5.7	3.8	8.6	5.8	1.23	0.21	21.37	32
Collar width at basis	3.5	2.5	4.4	3.5	0.50	0.08	14.39	40
Collar width at top	8.0	5.2	10.4	8.3	1.34	0.36	16.15	13
Microvilli number	22	20	24	22	2.83	1.41	12.86	2
Diameter nucleus	2.5	1.2	3.2	2.5	0.39	0.05	15.46	52
Diameter nucleolus	1.7	0.8	2.5	1.7	0.32	0.04	18.74	57
Flagellum length	8.5	6.9	9.8	8.6	0.95	0.28	10.97	10
Diameter vacuole	2.2	1.2	3.2	2.2	0.40	0.05	18.21	73
Stalk length	3.04	1.61	4.61	3.0	0.95	0.27	31.82	11
Longest food particle ingested	1.2	0.6	2.5	1.2	0.41	0.06	35.70	40

Abbreviations: Mean - arithmetic mean; Min - minimum; Max - maximum; SD - standard deviation; SE – standard error; CV % - coefficient of variation in %. All measurements in µm.

Supplement Table 1. Uncorrected pairwise distances between SSU rDNA genes of the Craspedida.

	Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1	*"Salpingoeca" fusiformis*																								
2	*Paramonosiga thecata*	0.157																							
3	*"Salpingoeca" euryoecia*	0.127	0.176																						
4	*"Salpingoeca" longipes*	0.192	0.189	0.216																					
5	*"Salpingoeca" ventriosa*	0.148	0.178	0.184	0.183																				
6	*"Sphaeroeca" leprechaunica*	0.200	0.212	0.215	0.211	0.189																			
7	*"Sphaeroeca" volvox*	0.184	0.205	0.213	0.215	0.193	0.045																		
8	*"Salpingoeca" infusionum*	0.201	0.183	0.208	0.015	0.185	0.223	0.230																	
9	*Monosiga brevicollis*	0.107	0.135	0.135	0.145	0.134	0.192	0.172	0.159																
10	*Codosiga botrytis*	0.205	0.207	0.232	0.224	0.218	0.185	0.190	0.226	0.187															
11	*Codosiga balthica*	0.138	0.149	0.164	0.158	0.157	0.207	0.193	0.176	0.090	0.199														
12	*Codosiga gracilis*	0.118	0.141	0.141	0.158	0.144	0.201	0.186	0.176	0.052	0.196	0.094													
13	*Salpingoeca napiformis*	0.098	0.137	0.131	0.158	0.130	0.175	0.158	0.176	0.083	0.184	0.123	0.095												
14	*Salpingoeca* sp_EU011930	0.109	0.141	0.144	0.166	0.150	0.190	0.176	0.182	0.095	0.184	0.115	0.099	0.091											
15	*Salpingoeca rosetta*	0.220	0.227	0.246	0.214	0.196	0.240	0.240	0.205	0.188	0.251	0.195	0.191	0.204	0.201										
16	*Salpingoeca urceolata*	0.118	0.153	0.144	0.170	0.153	0.193	0.178	0.191	0.092	0.191	0.117	0.098	0.101	0.085	0.209									
17	*Salpingoeca amphoridium*	0.127	0.166	0.151	0.220	0.174	0.210	0.193	0.227	0.127	0.206	0.152	0.128	0.113	0.118	0.243	0.129								
18	*Choanoeca perplexa*	0.133	0.150	0.147	0.164	0.154	0.207	0.196	0.190	0.062	0.205	0.105	0.021	0.109	0.113	0.203	0.110	0.140							
19	*"Desmarella" moniliformis*	0.107	0.158	0.113	0.191	0.150	0.200	0.192	0.203	0.114	0.202	0.151	0.126	0.110	0.116	0.226	0.128	0.128	0.135						
20	*"Monosiga" ovata*	0.063	0.145	0.120	0.175	0.129	0.190	0.179	0.193	0.094	0.188	0.119	0.105	0.087	0.092	0.206	0.099	0.110	0.116	0.083					
21	*Codosiga minima*	0.139	0.141	0.161	0.141	0.135	0.196	0.180	0.163	0.099	0.188	0.060	0.104	0.125	0.108	0.174	0.119	0.145	0.114	0.141	0.121				
22	*Codosiga* sp_JF706242	0.229	0.221	0.244	0.225	0.223	0.171	0.178	0.222	0.197	0.132	0.214	0.209	0.189	0.202	0.259	0.207	0.215	0.219	0.211	0.200	0.209			
23	*Codosiga* sp_JF706241	0.214	0.221	0.229	0.238	0.225	0.165	0.162	0.233	0.189	0.125	0.215	0.196	0.181	0.191	0.263	0.196	0.209	0.207	0.205	0.189	0.199	0.115		
24	*Codosiga* sp_JF706239	0.198	0.206	0.219	0.228	0.211	0.168	0.165	0.224	0.174	0.125	0.202	0.184	0.168	0.177	0.250	0.181	0.193	0.195	0.192	0.176	0.182	0.107	0.006	
25	Codosiga sp_JF706237	0.211	0.215	0.228	0.231	0.229	0.176	0.176	0.225	0.191	0.127	0.211	0.199	0.183	0.188	0.268	0.193	0.210	0.210	0.202	0.188	0.201	0.115	0.051	0.051

Chapter 3

A new group of acanthoecid related choanoflagellates from freshwater, sediment and soil

Abstract

The systematics of choanoflagellates is of great evolutionary interest as they are forming the sister group to Metazoa within the Opisthokonta. Recently, two orders of choanoflagellates have been described: Acanthoecida (loricates) and Craspedida (non-loricates). Molecular data mainly based on 18S rDNA, showed that on the one hand the phylogeny of loricate species is well-defined and families are monophyletic. On the other hand the phylogeny of the Craspedida is very controversial as no clear monophyletic clusters can be detected. Besides, some single well-documented freshwater Acanthoecida, representatives of this order of choanoflagellates are normally occurring in marine or brackish water habitats, whereas craspedid choanoflagellates are found in all types of aquatic habitats. Here, we present a new choanoflagellate group with several uncultured environmental clones including the new choanoflagellate genus *Acanthafallax* with the new species *A. monosigata* isolated from the River Rhine at Cologne, Germany. *A. monosigata* is showing craspedid morphology ("*Monosiga*-like"), though the sequencing of the 18S and 28S rDNA surprisingly revealed a phylogenetic position as a separated group related to the Acanthoecida. Here, the morphology and phylogeny and some ecological characteristics are discussed.

Introduction

Choanoflagellates (Choanomonada) are a group of small phagotrophic protists. Their phylogenetic position within the Opisthokonta as the sister group to Metazoa, placed them in the focus of evolution and systematic research (Carr et al. 2008; Cavalier-Smith and Chao 2003; King et al. 2008; Medina et al. 2003; Richter and King 2013; Ruiz-Trillo et al. 2008; Steenkamp et al. 2006). Choanoflagellates play an important microbial role as they act as filter feeders using their single apical flagellum producing water current and their collar of microvilli trapping many bacteria as feeding particles simultaneously (Boenigk and Arndt 2000, 2002; Pettitt et al. 2002). They are currently classified into two distinct orders: Acanthoecida and Craspedida Cavalier-Smith (1996). The order Acanthoecida is characterized by a basket-like lorica structure of silicified costae around the protoplast. Two monophyletic families are known: nudiform Acanthoecidae Norris emend. *sensu* Nitsche et al. (2011) and tectiform Stephanoecidae Leadbeater (2011) (Leadbeater 1979; 2008; Manton et al. 1981). According to literature, both families are frequent in marine habitats (Carr et al. 2008; Leadbeater 2008), whereas only few records of brackish and freshwater Acanthoecida exist: *Stephanoeca apheles* and *Diaphanoeca grandis* from north-eastern German lakes at salinities of about 3 PSU (Auer and Arndt 2001), *Stephanoeca arndtii* found in a freshwater lake on Samoa (Nitsche 2014) and *Acanthocorbis mongolica* of a freshwater Mongolian lake (Paul 2012).

In contrast to Acanthoecida, representatives of the order Craspedida are occurring in nearly all aquatic habitats and even soil. The order Craspedida is currently consisting of the family Salpingoecidae Kent emend. *sensu* Nitsche et al. (2011) with thecate and non-thecate species. A revised taxonomy of the Craspedida is still in need as no distinct monophyletic clusters within this order could yet be detected (Carr et al. 2008; chapter 2). Until recently, Craspedida were classified according to the presence or absence of the organic cell covering called theca or the ability to form colonies based on the descriptions by Saville Kent (1880-82). Representatives without a theca are named “*Monosiga*-like” species, those with a rigid theca surrounding the protoplast

are considered to be “*Salpingoeca*-like” species, and those without a theca but with a fine coat (glycocalyx) and often occurring as colonies are called “*Codosiga*-like” forms. Nonetheless, this classification might have to be reconsidered when more phylogenetic data by multigene analyses will be available (Nitsche et al. 2011).

In the present study we described, based on environmental sequences and one cultivated species, a new group of acanthoecid related choanoflagellates. All species originated from freshwater, brackish water, sediment or soil. Clustering within this group, we described a freshwater isolate (River Rhine, Cologne, Germany) with surprising craspedid morphology. We defined this new genus and species *Acanthafallax monosigata* using morphological and phylogenetic data (18S + 28S rDNA sequence data). In addition, we did autecological observations to characterize *Acanthafallax monosigata* regarding its unusual position of a freshwater, non-loricate species clustering close to the Acanthoecida.

Material and Methods

Material collection and cultivation

The species was collected on 03/09/98 from the Rhine River at Cologne, Germany (50° 54’N/6° 58’E). 500ml of surface water were taken in a sterile polyethylene bottle. Aliquots were transferred to cell culture flasks (50 ml, Sarstedt, Nümbrecht, Germany). As food source for the choanoflagellates, a sterilized wheat grain was added to support the growth of autochthonous bacteria. The culture flasks were kept at 10°C and a 12/12h day/night cycle. Each week, the cultures were observed by light microscopy (Zeiss Axiovert S 100). To obtain a clonal culture of the species, we diluted the raw cultures containing choanoflagellates using the liquid aliquot method (Butler and Rogerson 1995) and using a micromanipulator (Patchman MP2, Eppendorf, Hamburg, Germany) to transfer single cells to culture flasks. Wright’s Chu

medium (Guillard and Lorenzen 1972) was used for cultivation. The culture is available from the author upon request.

Video microscopy

For the examination of the species morphology, an inverted microscope system was used. We prepared Petri dishes with coverslips as base for cultivating and observing the clonal culture by a Zeiss Axio Observer with a 100x/1.4 NA oil immersion objective (DIC) and a water immersion condenser. We took video images using a black/white analogous Hamamatsu C6489 camera and an Allen Video Enhanced Contrast (AVEC) system (Hamamatsu, Argus-20) for noise reduction and contrast enhancement (for details of the setup see Stoupin et al. 2012). The video analysis was carried out frame by frame. Images were analysed using the VirtualDub (www.virtualdub.org), ImageJ (Abramoff et al. 2004), and AviStack (www.avistack.de) software. For the time-lapse analysis we used the hssVss (www.hssvss.com) software and observed the specimens for several hours taking a picture every five seconds.

Electron microscopy

For scanning electron microscopy we used a fixation ratio of one to one with Bouin’s fixative and 1% osmium tetroxide (final concentration) at 4°C for 30 min. Three parts saturated picric acid and one part buffered formaldehyde (38%) with 2% glacial acetic acid were added immediately before fixation to the Bouin`s fixative. To reach a final concentration of 0.1-0.2%, Glutaraldehyde was added to the final solution. The sample was kept in the culture flask and was dehydrated in an ethanol series comprising 30%, 50%, 60%, 80%, 90%, 96% and pure ethanol. We washed the sample two times with the corresponding ethanol concentration and left it finally for 10 min in each solution. Afterwards, a 50:50 hexamethyldisilazane (HMDS)-ethanol solution was utilized for 15 min followed by pure HMDS for 15 min as a substitute for

critical point drying (Nitsche and Arndt 2008). After this procedure, the sample was allowed to dry. We cut the bottom of the flask to appropriate size and stuck it to a sample holder**.** The SEM sample was sputter coated with a 120Å layer of gold before examination by SEM (FEI Quanta 250 FEG).

Molecular biological analysis

The DNA extraction was performed using the Quick gDNA^{TM} Mini Prep (Zymo Research Corporation, CA, USA). The 18S rDNA was amplified using the following primer combinations (0.1mM concentration): 42 F (5'-CTCAARGAYTAAGCCATGCA-3') + 18S-Rev-1 (5'-ACCTACGGAA-ACCTTGTTA-CG-3'), 82F (5'-GAAACTGCGAA-TGGCTC-3') + 1630R (5'-CGACGGGCG-GTGTGACAA-3'), and 590F (5'- CGTAA-TTCCAGCTCCAATAGC-3') + 1300R (5'-CACCAACTAAGAACGGCCATGC-3') and a PCR Mastermix (2x) (Genaxxon Bioscience, Ulm, Germany) for all reactions. For amplification the mixture was initially heated to 96°C for 2min, followed by 32 cycles of 95°C for 30s, 48°C for 30s, 52°C for 30s, and 72°C for 2min, finished by a final elongation for 7 min at 72°C. Purification of the PCR products was carried out using the PCR Purification Kit (Bioscience, Jena, Germany) and both strands of the purified products were sequenced. We tested all strands for consistency. The 28S rDNA amplification was performed according to Carr et al. (2008). The amplification, PCR purification and sequencing were carried out as described for single-cell PCR. All strands were tested for consistency. The LSU rDNA amplification was performed according to Carr et al. (2008).

Phylogenetic analysis

Alignments of the 18S and 28S rDNA sequences were carried out using Kalign (Lassmann and Sonnhammer 2005) and corrected manually. We used 44 choanoflagellate taxa and up to 4777 unambiguously aligned nucleotides (18S and partial 28S rDNA) to perform concatenated trees by maximum likelihood (ML) and

Bayesian inference (BI) analysis. The ML tree was calculated using RAxML GUI 1.3 (Silvestro and Michalak 2012) with the GTRCAT model as suggested by the authors. We utilized a two-taxa clade of Ichthyosporea (i.e. *Amoebidium parasiticum* (SSU:Y19155/LSU:EU011932), *Ichthyophonus hoferi* (SSU:U43712/LSU:AY026370)) and a nine-taxa metazoan clade (i.e. *Beroe ovata* (SSU:AF293694/LSU:AF293694), *Haliclona* sp. (SSU:KC902267/LSU:KC869594), *Hydra magnipapillata* (SSU: HQ392522/LSU:HQ392528), *Leucosolenia* sp. (SSU:AF100945/LSU:AF100945), *Mnemiopsis leidyi* (SSU:AF293700/LSU:AF293700), *Nematostella vectensis* (SSU:AF254382/LSU:AY345871), *Suberites domuncula* (SSU:AJ620112/LSU:AJ620 112), *Sycon calcaravis* (SSU:D15066), *Trichoplax* sp. (SSU:Z22783/LSU:AY652581)) as outgroup according to Nitsche et al. (2011). The Bayesian analysis was calculated by MrBayes v3.2.1 (Ronquist and Huelsenbeck 2003) using a GTR model and a four-category gamma distribution correcting for rate variation among sites. For each portion, MrBayes computed the parameters for tree topology, branch length, individual nucleotide substitution rates, nucleotide frequency, proportion of invariable site, and gamma distribution shape parameter. The analysis was run for 1,000,000 generations with an average standard split deviation <0.007528 and a “burnin” of 250, before computing posterior probabilities.

The new sequences are available in GenBank under the Accession numbers KJ957795 (18S rDNA) and KJ957794 (28S rDNA) and the alignments are obtainable from the author upon request.

Autecological observations

The ecological requirements of *Acanthafallax monosigata* regarding two parameters (salinity [PSU (Practical Salinity Units)] and sodium metasilicate (Na_2SiO_3 x 5 H_2O)) were tested in batch incubations. The cells were observed via the video-microscopy method described above. For the salinity tolerance experiments, three 2ml replicates were gradually adapted to higher salinities (0.5 PSU/day) by adding artificial seawater with a salinity of 100 PSU (per litre deionized water: 80.5g NaCl, 1.92g KCl, 15.76g

$MgCl_2$ x $6H_2O$, 19.8g $MgSO_4$ x $7H_2O$, 4.15g $CaCl_2$ x $2H_2O$, 0.286g KNO_3, 0.028g K_2HPO_4 x $3H_2O$). The salinity value was controlled using a refractometer. The cell reactions regarding higher salinity levels were observed by the above described microscopy method and time-lapse analysis. We used a Petri dish in which the salinity level could get increased stepwise while observing the same cells for the whole time. For testing the ability of lorica production under higher sodium metasilicate content, 180 µM Na_2SiO_3 x 5 H_2O was supplied to three 20 ml parallels (compare Leadbeater 1985, 1989) and observed every 24 hours for several weeks.

Results

We isolated, sequenced (18S and 28S rDNA) and established a new genus with one type species showing craspedid morphological features, but being positioned within a new clade close to acanthoecids. The morphometric data of *Acanthafallax monosigata* are shown in Table 1. Additionally, we carried out autecological experiments to characterize the new species and to reveal a hint regarding the ecological specificity of the new.

Phylogenetic analysis

Based on partial 18S and 28S rDNA of *Acanthafallax monosigata* we generated a concatenated phylogeny (Fig. 1). The topology of previous studies (Carr et al. 2008; Nitsche et al. 2011; Wylezich et al. 2012) was basically recovered. Some new phylogenetic relationships became obvious due to the addition of sequences of *Acanthafallax* and uncultured environmental clones.

According to the analysis, the 44 choanoflagellates and 9 metazoan taxa were distinctly branching with strong support (100% mlBP [maximum likelihood bootstrap percentage] and 1.00 biPP [Bayesian interference posterior probability]). The monophyletic clustering of the order Craspedida (96% mlBP; 0.99 biPP) and

Acanthoecida together with the new group (99% mlBP; 0.99 biPP) respectively was highly confirmed. We received moderate mlBP evidence for the monophyly of both families of Acanthoecida (tectiform Stephanoecidae: 57% mlBP; nudiform Acanthoecidae: 100% mlBP). Hence, the addition of the *Acanthafallax monosigata* sequence and uncultured clones revealed a new clade (clade A) as a sister group to Acanthoecida (100% mlBP; 0.99 biPP). *Acanthafallax* is clustering with 97% mlBP; 0.99 biPP together with AY821949, a clone isolated from anoxic sediments of a suboxic pond (Šlapeta et al. 2005).

The phylogeny based on an 18S single-gene analysis is not shown as the monophyly of Acanthoecidae and Stephanoecidae, respectively, was not recovered. However, this phylogeny can be provided by the author upon request.

Table 1. Morphometric data of *Acanthafallax monosigata*.

Character	Mean	Min	Max	Median	SD	SE	CV %	n
Body length	3.45	2.53	4.38	3.28	0.49	0.08	14.17	34
Body width	3.18	2.23	4.26	2.94	0.64	0.10	20.08	36
Collar length	5.95	3.8	7.6	5.82	0.89	0.18	14.92	22
Collar width at basis	2.38	1.84	3.11	2.3	0.32	0.06	13.44	25
Collar width at top	5.41	3.69	8.06	4.84	1.61	0.43	29.85	13
Microvilli number	20.33	16	30	20	3.89	1.08	19.14	12
Diameter nucleus	1.56	1.27	1.96	1.5	0.23	0.08	14.65	7
Diameter nucleolus	0.89	0.69	1.04	0.92	0.11	0.04	12.37	7
Flagellum length	11.33	8.75	13.83	11.24	2.12	0.79	18.76	6
Diameter vacuole	1.35	0.81	2.3	1.38	0.27	0.04	20	40
Longest food particle ingested	0.69	0.46	0.92	0.69	0.16	0.04	22.76	16

Abbreviations: Mean - arithmetic mean; Min - minimum; Max - maximum; SD - standard deviation; SE – standard error; CV % - coefficient of variation in %. All measurements in µm.

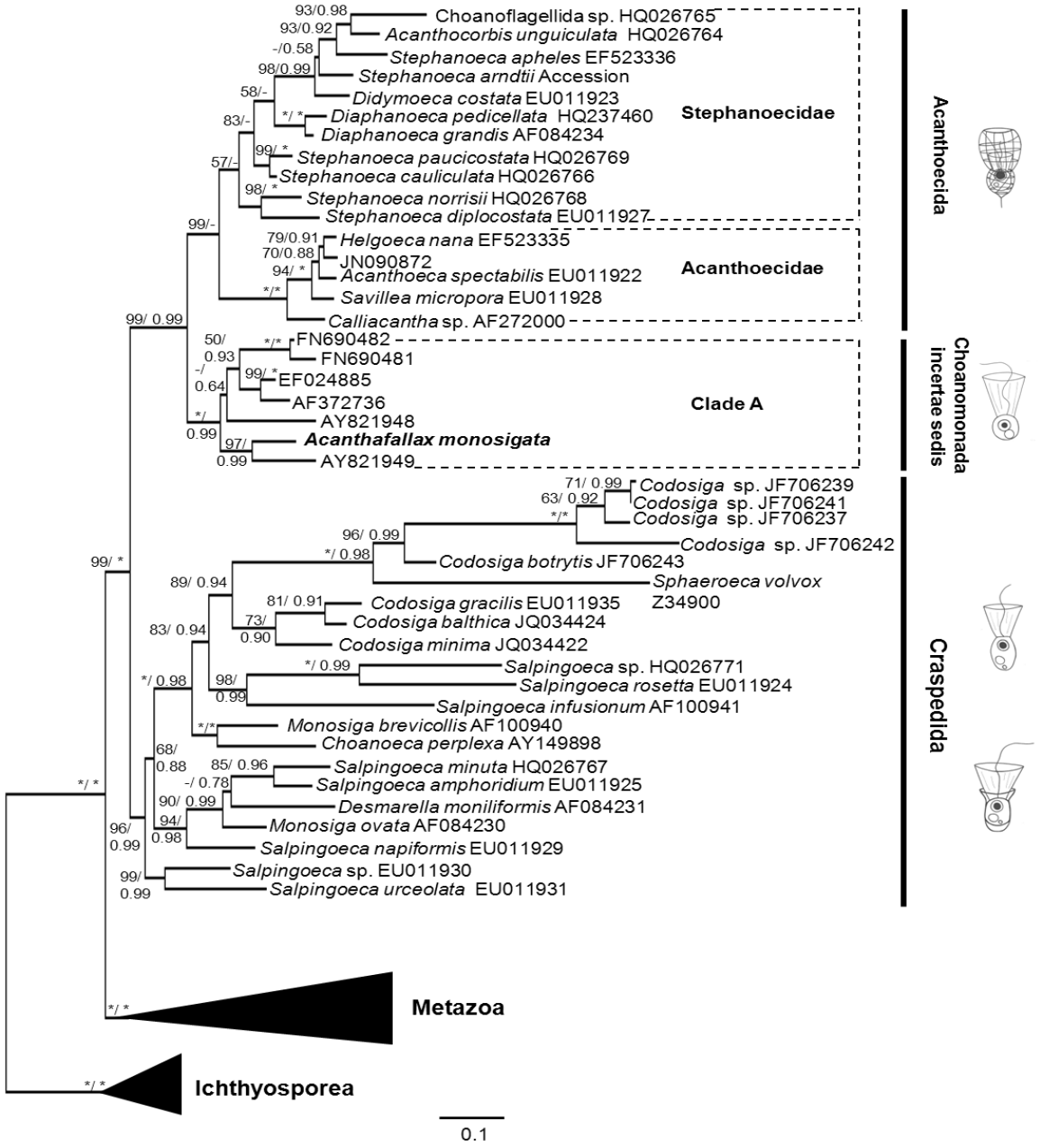

Figure 1. Concatenated maximum likelihood phylogeny of 18S and 28S rDNA choanoflagellate sequences (4777 nt). The newly sequenced species *Acanthafallax monosigata* is marked by bold letters. RAxML/BI support values are offered at each node. 100 % RAxML bootstrap percentage support (mlBP) and 1.00 BI posterior probabilities (biPP)

are indicated by *. Unrecovered topologies and support values under 50% mlBP are denoted by -. Numbers of nucleotide substitutions per site are indicated by the scale bar in the lower middle.

Morphological description

Genus *Acanthafallax* Jeuck, Nitsche and Arndt gen.nov.

Diagnosis: Small, ovoid to roundish cells, uninucleate with one centrally and anteriorly positioned flagellum, lacking lorica production. Flagellum surrounded by a collar consisting of microvilli. Phagotrophic filter feeders. Freshwater, slightly brackish water, sediments and soil. Genetically (18S and 28S rDNA) distant from morphologically similar craspedid genera.
Type species: *Acanthafallax monosigata* Jeuck, Nitsche and Arndt.
Etym. *acantha* Gk. from Ancient Greek “akantha” meaning “spine” + *fallax* L. deceptive, because the *Monosiga*-like morphology is misleading to Craspedida.

***Acanthafallax monosigata* Jeuck, Nitsche and Arndt** sp. nov. Figs.: 2 A-F, type strain: HFCC 46 (River Rhine, Cologne), type sequences: 18S rDNA: KJ957795, 28S rDNA: KJ957794.

Diagnosis: Freshwater “*Monosiga*-like” species, about 3.4 x 3.2 µm in size. The collar has a length of about 6 µm with about 19 microvilli. The flagellum is about 11.3 µm long.

Description: The roundish species has a body length of about 2.5-4.4 µm (3.4 µm on average) and a body width of about 2.2-4.3 µm (3.2 µm on average). The acronematic flagellum has a length of 8.7-13.8 µm (11.3 µm on average) (Fig. 2A). The collar is about 1.8-3.1 µm (2.4 µm on average) wide at the basis and about 3.7-8 µm (5.4 µm on average) long at the top. The species possesses about 16-30 (20.3 on average) relatively short microvilli. The diameter of the nucleus is about 1.3-2 µm (1.5

µm on average) and the nucleolus is about 0.7-1 µm (on average 0.9 µm) in diameter. The feeding vacuole with an average diameter of about 1.3 µm is located posteriorly (Fig. 2D, G). Food particles transported along the microvilli (tentacles) had an average size of 0.7 µm (Fig. 2C, F), they are ingested along the cell surface at the base of the collar (Fig. 2F, see also Boenigk and Arndt 2000 for *Monosiga ovata*). Cyst formation with a thick cyst wall occurs.
Etym. *monosigata* L. latinized because showing a *Monosiga*-like appearance.

Remarks: The roundish protoplast of *Acanthafallax monosigata* is relatively similar to *Monosiga brevicollis* Ruinen (1938), *Monosiga consociatum* Saville Kent (1880), and *Monosiga ovata* Saville Kent (1880) and the feeding mechanism is similar to *Monosiga ovata* (see above). However, its phylogenetic position, not within the Craspedida and not within the previously described Acanthoecida, is unique.

We tested the tolerance of *Acanthafallax monosigata* regarding a salinity gradient. The species was able to survive stepwise adaptation (steps of 0.5 PSU per day) up to a salinity of 1.5 PSU. A further increase of salinity and a direct addition of 1 PSU water caused direct cell shrinkage and cell lysis (see Fig. 2E). While treating the cysts of *Acanthafallax monosigata* with a stepwise increase of 1 PSU, the cysts stayed intact up to salinity levels of 30 PSU. Cysts could also be directly transferred from 30 PSU to 0 PSU without damage. However, we were not able to check viability of cysts after treatment.

Furthermore, we tried to stimulate lorica production of *Acanthafallax monosigata* by cultivating the species in silicate-rich medium. However, no lorica production was observed cultivating the species in a medium containing up to 180 µM silicate per litre.

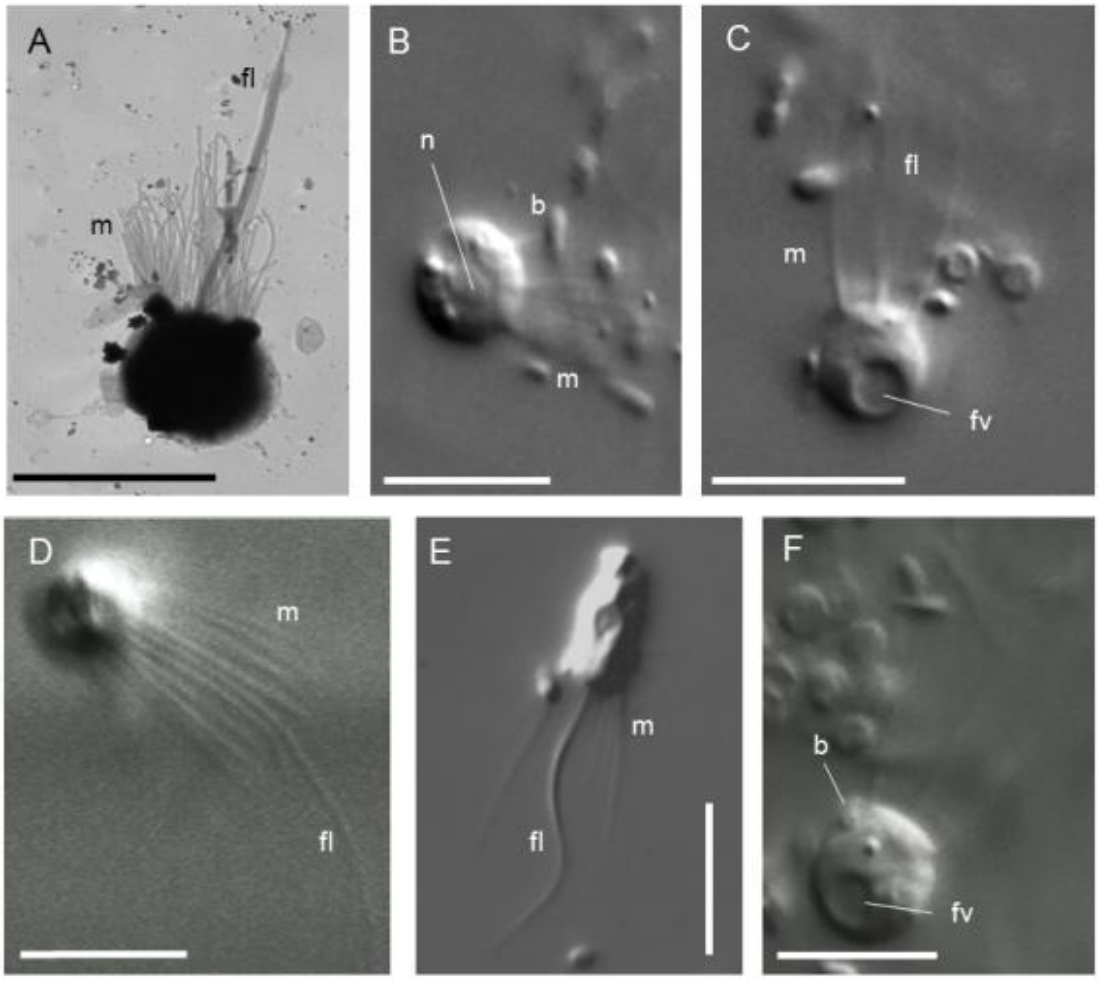

Figure 2. General morphology of *Acanthafallax monosigata*

A: Electron micrograph of the species; **B:** Fine structure of the cell with nucleus; **C:** Fine structure of the cell with feeding vacuole; **D:** Microvilli and flagellum; **E:** Protoplast and flagellum treated with artificial seawater of 1 PSU; **F:** Ingested bacterium at cell surface. Scale: 5µm; Abbreviations: b – bacterium; fl – flagellum; fv – feeding vacuole; m - microvilli; n – nucleus.

Discussion

We discovered a new group of acanthoecid related choanoflagellates consisting of *Acanthafallax monosigata* and several uncultured clones. We refrain from naming this new clade (A) as a new family before more species and information will be available in future. The uncultured clones are special with regard to their occurrence in freshwater or terrestrial habitats including suboxic/anoxic environments (Table 2). None of the uncultured clones could be described morphologically. Our study emphasizes the importance of the combination of both morphological and molecular techniques (DeSalle et al. 2005; Will and Rubinoff 2004). Šlapeta et al. (2005) already speculated that genotypes AY821949 and AY821948 might be morphologically distant from known choanoflagellates as the sequences were significantly different and distantly related to other groups of choanoflagellates. With the identification and characterisation of *Acanthafallax monosigata,* a classification of those uncultured sequences is possible now. Studying species belonging to this group might be very interesting regarding their basic phylogenetic position and the different habitat preference compared to other acanthoecid choanoflagellates. Most of them are freshwater isolates originating from suboxic or anoxic conditions. A transition from marine to freshwater has been recorded for a few loricated acanthoecid choanoflagellates of the genus *Stephanoeca* (Nitsche 2014) and *Acanthocorbis* (Paul 2012). However, a freshwater acanthoecid related choanoflagellate with craspedid morphology has, according to our knowledge, previously never been detected. *Acanthafallax monosigata* is showing craspedid *"Monosiga*–like" morphological characteristics although clustering close to acanthoecid choanoflagellates. We carried out experiments to test whether this species possess the ability to tolerate high salinities and to produce a lorica.

Table 2. Origin of environmental sequences of the new group of choanoflagellates.

Sequence accession number	Origin of sequence	Environmental conditions	Reference
AF372736	Bloomington, IN, USA	Freshwater sediment in Lake Lemon, 3-5 cm depth in loose sediment (pH 7.5, 28°C)	Dawson and Pace (2002)
AY821948	Campus of Université Paris-Sud	Suboxic pond (circa 15m diameter, pH 6.5, water temp. 11.5°C, 0.38 (bottom) to 2.4 mg l^{-1} (surface) oxygen	Šlapeta et al. (2005)
AY821949	Campus of Université Paris-Sud	Suboxic pond (circa 15m diameter, pH 6.5, water temp. 11.5°C, 0.38 (bottom) to 2.4 mg l^{-1} (surface) oxygen	Šlapeta et al. (2005)
EF024885	Rhinelander, WI, USA	FACE experiment, soil cores, planted with trembling aspen, exposed to elevated CO_2 (560 p.p.m.)	Lesaulnier et al. (2008)
FN690481	Baltic Sea (Gulf of Bothnia: 62°43.9'N, 19°55.7'E)	Sea ice samples, salinity about 3 PSU	Majaneva et al. (2012)
FN690482	Baltic Sea (Gulf of Bothnia: 62°43.9'N, 19°55.7'E)	Sea ice samples, salinity about 3 PSU	Majaneva et al. (2012)

As a result mentioned above, *Acanthafallax monosigata* was not able to tolerate salinity values >1.5 PSU when stepwise adapted. Direct treatment with 1.0 PSU even caused direct cell extinction. Cysts seem to survive salinity treatments. It is remarkable that cyst formation has up to our knowledge never been reported for acanthoecid choanoflagellates. *Acanthafallax monosigata* was not able to build a lorica under silica-rich medium conditions; Acanthoecida as *Stephanoeca diplocostata* are able to loose and rebuild the lorica at differing silicate concentrations (compare Leadbeater 1985, 1989). Preliminary growth experiments under suboxic/anoxic conditions indicated lower though positive growth rates of *Acanthafallax*. Future experiments and isolation of additional members of this group might help to clarify whether the tolerance of low oxygen concentrations or anoxic conditions might be a specific character of this new group of choanoflagellates.

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Chapter 4

A six-gene phylogeny provides new insights into choanoflagellate evolution

Abstract

Recent studies have shown that molecular phylogenies of the choanoflagellates (Choanomonada) are in disagreement with their traditional taxonomy and that Choanomonada requires considerable taxonomic revision. Furthermore, phylogenies suggest that the morphological and ecological evolution of the group is more complex than has previously been recognized. Here we address the taxonomy of the major choanoflagellate order Craspedida, by erecting three new genera. The new genera are shown to be morphologically, ecologically and phylogenetically distinct from other choanoflagellate taxa. Furthermore, we name five novel craspedid species, as well as formally describe ten species that have been shown to be misidentified.

The revised phylogeny, augmented by newly generated transcriptome data, provides insights into the morphological and ecological evolution of the choanoflagellates. Additionally, at the genome level we examine the distribution of two closely related translation GTPases, EF-1A and EFL, which are required for protein synthesis. Mapping the presence and absence of the genes onto the phylogeny highlights multiple events of gene loss in the choanoflagellates.

Introduction

The choanoflagellates are a ubiquitous group of aquatic bacterivore filter feeders (Arndt et al. 2000) and interest in their evolutionary biology has increased due to their recognized position as the sister-group to Metazoa in the eukaryotic supergroup Opisthokonta (Adl et al. 2012; Carr et al. 2008; Ruiz-Trillo et al. 2008). The opisthokonts are divided into two major lineages, these being Holozoa, comprising Metazoa and the protistan Choanomonada, Filasterea, Ichthyosporea plus Corallochytrea, and Nucletmycea (sometimes referred to as Holomycota) comprising Fungi and the nuclearioid amoebae (Adl et al. 2012).

It has long been acknowledged that the taxonomy of the choanoflagellates is in need of significant revision (Cavalier-Smith and Chao 2003; Medina et al. 2003; Carr et al. 2008; Leadbeater et al. 2008; Nitsche et al. 2011; Stoupin et al. 2012). Choanoflagellate taxonomy has, in the past, been based upon morphological characters; in particular the external covering of the cell defined the three traditionally recognized families. Choanoflagellates possessing a solely organic cell cover were split into two families, Salpingoecidae Saville Kent (which possessed a rigid theca) and Codonosigidae Saville Kent (often called ‘naked’ choanoflagellates, which possessed a fine mucilaginous cover that is referred to as the glycocalyx); however, in molecular phylogenies neither group was recovered as monophyletic (Cavalier-Smith and Chao 2003; Medina et al. 2003). Nitsche et al. (2011) showed that Codonosigidae is polyphyletic within Salpingoecidae and therefore synonymized the former with the latter within the order Craspedida Cavalier-Smith. The thecae of salpingoecids are found in a variety of morphologies; the most commonly observed are the flask (exemplified by *Choanoeca perplexa*, see Leadbeater, 1977), the cup (exemplified by *Salpingoeca rosetta*, see Dayel et al. 2011) and the tube (exemplified by *Salpingoeca tuba*, see Nitsche et al. 2011). Nitsche et al. (2011) also formally described two families of loricate choanoflagellates which produce cage-like silica baskets. The nudiform taxa were assigned to the Acanthoecidae Ellis *sensu* Nitsche et al. (2011),

whilst tectiform taxa were assigned to a new family, Stephanoecidae Leadbeater (2011).

We present here a molecular phylogeny containing 42 choanoflagellate species, created using a six-gene dataset. The six genes are 18S small-subunit ribosomal DNA (SSU), 28S large-subunit ribosomal DNA (LSU), 90-kilodalton heat shock protein (hsp90), alpha-tubulin (tubA), elongation factor-1A (EF-1A, formerly EF-1α) and elongation factor-like (EFL). The revised phylogeny provides the basis to revise aspects of choanoflagellate taxonomy at the generic level; in particular we have amended the genus *Codosiga*. The first probable description of a *Codosiga* species was that of *Codosiga botrytis* Ehrenberg, prior to the recognition of choanoflagellates as a group, under the name *Epistylis botrytis* (Ehrenberg 1831, 1838). The species was subsequently described in other works as *Anthophysa solitaria* (Fresenius 1858) and *Codosiga pulcherrima* (James-Clark 1867). Bütschli (1878), Saville Kent (1878) and Stein (1878), working contemporaneously, revisited Ehrenberg's (1838) description of *Epistylis botrytis* and all decided that it was synonymous with James-Clark's (1867) *C. pulcherrima*. *Codosiga* currently comprises approximately 20 species of non-thecate craspedids, which form multi-headed stalked colonies. Most described taxa inhabit freshwater, with only four species (*C. balthica* Wylezich and Karpov, *C. cymosa* Saville Kent, *C. gracilis* Saedeleer, and *C. minima* Wylezich et Karpov) recognized as marine (including brackish waters and therefore defined as >0.5 parts per thousand). We have included nine recognized members of the currently defined genus *Codosiga* in our phylogeny and our results show that they are found in two distantly related marine and freshwater groups. The genus *Codosiga*, as it currently stands, is polyphyletic. The marine species are therefore re-assigned to a new genus, *Hartaetoesiga*, and we re-describe *Codosiga* here. Furthermore, we erect a new genus, *Stagondion*, for a craspedid that possesses an ovoid theca. We also create a new genus and formally describe a naked craspedid erroneously deposited at the American Type Culture Collection (ATCC) under the name *Monosiga ovata*, which is morphologically and ecologically distinct from other *Monosiga* species. Nitsche et al. (2011) highlighted that a further four choanoflagellate species held in ATCC had been

misidentified. We expand on this finding here and describe the species, as well as five novel species of craspedid.

The four-gene phylogenetic analysis of Carr et al. (2008) produced new insights into the evolution of choanoflagellates, but was hindered by only containing 16 species. The 42-taxa phylogeny presented here provides unprecedented insights into the morphological, ecological and genomic evolution of the choanoflagellates. The phylogeny is consistent with single origins of the tube, cup and flask theca morphologies, although multiple origins of the latter two cannot be discounted. Furthermore, all of the freshwater species in our phylogeny cluster together in a single group, revealing a major freshwater radiation in the ancestrally marine choanoflagellates. Environmental SSU sequences however indicate that multiple freshwater incursions may have occurred during choanoflagellate evolution.

EF-1A is a major component of the eukaryotic protein synthesis machinery. Due to its importance in protein translation and its involvement in multiple additional pathways (Gaucher et al. 2001), EF-1A was considered an essential and ubiquitously distributed protein. It was therefore a considerable surprise when it was discovered that a number of eukaryotic taxa lacked EF-1A (Keeling and Inagaki 2004). Those species which do not possess EF1-A have been shown to encode a paralogous GTPase. This protein family, EFL or Elongation Factor-like, has a punctate distribution within eukaryotes and phylogenies based on EFL sequences are not congruent with accepted species phylogenies (Keeling and Inagaki 2004, Noble et al. 2007). It has been speculated that EFL has undergone repeated rounds of lateral transfer into new hosts and, on occasion, replaced the endogenous EF-1A (Kamikawa et al. 2010a). EFL has previously been sequenced from representatives of Fungi, Choanoflagellatea and Ichthyosporea (Keeling and Inagaki 2004; Ruiz-Trillo et al. 2006; Noble et al. 2007; Marshall and Berbee 2010), although each of these lineages also contains taxa which encode EF-1A. EFL appears to be absent from metazoans, with all studied species encoding EF-1A. Within the choanoflagellates EF-1A has been shown to be present in three freshwater craspedids, these being

Codosiga botrytis, ATCC 50635 and ATCC 50153 (Steenkamp et al. 2006; Paps et al. 2013), whilst EFL has been found in *Monosiga brevicollis* ATCC 50154 and *Salpingoeca rosetta* ATCC 50818 (Noble et al. 2007). We show here that EF-1A is absent from the genomes of many choanoflagellate species, with 17 out of 22 examined taxa encoding EFL. Phylogenetic analyses of the EFL and EF-1A families show that both genes were present in the genome of the last common ancestor (LCA) of the extant choanoflagellates and that each gene has undergone multiple losses in the group.

Material and Methods

Isolation of choanoflagellate species and rDNA gene sequencing

Codosiga hollandica was isolated in June 2007 from a fresh water pond on Madeira. *Salpingoeca limnea* was isolated in August 2012 from glacial lake water on the island of Greenland. *S. calixa* was isolated from McKenzie Bay, Rangetoto Island, New Zealand in December 2010. *S*. *oahui* were isolated in May 2011 from a freshwater pond on the island of O`ahu, Hawaii. *Stagondion pyriformis* was isolated from Bálos Lagoon, Crete in August 2010. DNA amplification was performed using single cell PCR (Nitsche and Arndt 2008) applying the 28S large subunit ribosomal DNA (LSU) primers described in a previous study (Carr et al. 2008). The sequencing of LSU was performed using Big Dye-Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Weiterstadt, Germany) in accordance with the manufacturer's instructions. Both strands obtained were tested for consistency.

Cultured *Codosiga gracilis* (ATCC 50454) cells were grown in five 150 mm Petri dishes each containing ATCC Medium 1525 (artificial seawater cereal grass medium; https://www.atcc.org/Attachments/2750.pdf) for 5 weeks. Cells were collected with a scraper and centrifuged at 3220 x g at 4º C for 20 minutes, followed by aspiration of the supernatant and resuspension in lysis buffer (10 mM Tris, 0.1 µM EDTA, 0.0005% SDS, pH 8). Genomic DNA was extracted with a standard phenol/chloroform protocol (treatment with 20 µg/ml RNase A and 100 µg/ml Proteinase K followed by repeated

rounds of phenol:chloroform extraction and finally precipitation with 10M ammonium acetate and ethanol). PCR of 18S small subunit ribosomal DNA (SSU) ribosomal sequence was performed with the proofreading enzyme Pfu from Agilent and universal eukaryotic ribosomal primers 18S_1F (5' AACCTGGTTGATCCTGCCAGT 3') and 18S_1528R (5' TGATCCTTCTGCAGGTTCACCTAC 3') using the following program: 94º C for 3 minutes, 35 cycles of: 94º C for 30 seconds, 52º C for 30 seconds, 72º C for 3 minutes 30 seconds, followed by a final extension at 72º C for 10 minutes. PCR products were separated by gel electrophoresis and extracted with the QIAquick Gel Extraction Kit from Qiagen, followed by cloning using the TOPO TA Cloning vector from Invitrogen, both following the manufacturer's protocol. A single clone was selected and Sanger sequencing reads were generated using two primers within the vector sequence: M13F (5' GTAAAACGACGGCCAGTG 3') and M13R (5' CAGGAAACAGCTATGACC 3'). Internal sequence was generated using the pan-choanoflagellate 18S sequencing primers 18S_564F (5' AATTCCAGCTCCAATAGC 3') and 18S_1205R (5' ATGTCTGGACCTGGTGAG 3'). Sequence reads were base called using phred version 0.0210425.c (Ewing and Green 1998; Ewing et al. 1998) with default parameters, and aligned using FSA version 1.15.0 (Bradley et al. 2009) with default parameters.

A single 150mm Petri dish of ATCC 50964 (deposited under the name *Monosiga gracilis*) containing ATCC Medium 1525 was grown for a period of 2 days, collected with a cell scraper and centrifuged at 3220 x g at 4º C for 20 minutes. Following aspiration of the supernatant, cells were flash frozen in liquid nitrogen and stored at -80º C. Nucleic acid was extracted from the cell pellet using the RNAqueous Kit from Ambion following the manufacturer's protocol. Because the columns used in the RNAqueous protocol bind both RNA and genomic DNA, we used an aliquot of the extracted total nucleic acid for PCR on genomic DNA. PCR and all subsequent cloning and sequencing steps were performed identically to *Codosiga gracilis*. The SSU sequence of ATCC 50964 showed 99.8% identity to the published SSU of *C. balthica,* isolated from the Gotland Deep, Baltic Sea (Wylezich et al. 2012). This demonstrates

that ATCC 50964 is a North American isolate of *C. balthica* and disproves the endemism of this taxon as proposed by Wylezich et al. (2012).

Cultured ATCC 50959 (deposited under the name *Salpingoeca gracilis*) cells were grown in five 75 cm^2 tissue culture flasks containing ATCC Medium 1525 for 6 weeks. Cells were collected with a cell scraper, passed through a 40 µm filter and centrifuged at 3220 x g at 4º C for 20 minutes. Following aspiration of the supernatant, cells were flash frozen in liquid nitrogen and stored at -80 ºC. Nucleic acid extraction, PCR, cloning and sequencing were performed identically to *C. gracilis and* ATCC 50964, with one modification. Additional SSU sequence was generated to bypass an internal polynucleotide repeat by sequencing the cloned PCR product with the following three ATCC 50959-specific primers: Sg_18S_1 (5'-CCTTCAGACGAATCATCGAG-3'), Sg_18S_2 (5'-TGAGAACAAACCGCCAAAG-3') and Sg_18S_3 (5'-AATG –CCTGCTTTGAACACTC-3').

Transcriptome data

We augmented our data from PCR and sequencing by searching transcriptome data from 19 choanoflagellate species (Richter et al. in preparation). For SSU, LSU, hsp90 and tubA, we downloaded all available choanoflagellate data from GenBank and built multiple sequence alignments using FSA (Bradley et al. 2009) with default parameter values. We then removed unaligned regions using Gblocks (Talavera and Castresana 2007) with allowed gap positions set to “half” and all other parameter values set to their most permissive. Using the resulting file, we built HMMs using hmmbuild from the HMMER 3.0 package (http://hmmer.org/) with default parameter values. We searched each species' assembled transcriptome and its reverse complement using hmmsearch, also from the HMMER 3.0 package, with default parameter values. We chose the contig with the lowest E value as the representative sequence for that species. If there were multiple contigs with the same lowest E value, we chose the longest of those contigs. HMM profiles were created for EF-1A and EFL using MAFFT

6.935 (Katoh et al. 2002) nucleotide alignments, each generated from eight genes (see Supplement Table 1).

Phylogenetic analyses

The phylogeny of the choanoflagellates was analysed using partial sequences from SSU, LSU, hsp90 and tubA, EFL and EF-1A (Supplement Table 2). For each gene, DNA sequences from all species were aligned in MAFFT and then edited by eye to minimize insertion-deletion events.

The concatenated, six-gene, 9436 bp alignment was analysed using maximum likelihood and Bayesian inference methods. For both analyses the alignment was divided into separate partitions for ribosomal DNA, 1st and 2nd codon positions, as well as 3rd codon positions. All parameters for the phylogenetic analyses were estimated by each program. The maximum likelihood analysis was performed with RAxML 7.2.6 (Stamatakis 2006) using the GTRCAT model, as recommended by the program author. The analysis was initiated with 100 maximum parsimony trees and bootstrapped with 1,000 replicates. The Bayesian analysis was performed using MrBayes 3.1.1 (Ronquist and Huelsenbeck 2003) and run using a GTR+I+Γ model and a four-category gamma distribution to correct for among site rate variation. The search consisted of two parallel chain sets run at default temperatures with a sample frequency of 100 and run so that the average standard deviation of split frequencies dropped below 0.01. The analysis consisted of 2,000,000 generations, with a burnin of 5,000 before calculating posterior probabilities. The choanoflagellates were rooted with a two-taxa ichthyosporean clade and an eight-taxa metazoan clade.

Predicted amino acid sequences of EF-1A and EFL were recovered from GenBank using both keyword searches and BLASTp similarity searches with conceptual choanoflagellate protein sequences. Sequence recovery for EF-1A was restricted to Opisthokonta, whereas EFL sequences were recovered from all available eukaryotic groups. Alignments for each protein family were created using MAFFT and edited by eye. ProtTest 3.2.2 (Abascal et al. 2005) indicated that the LG+I+Γ+F (Le and Gascuel

2008) was the most appropriate amino acid substitution model for both EF-1A and EFL. Maximum likelihood phylogenies for both protein families were created using RAxML GUI 1.3 (Michalak 2012). Each analysis was performed with 25 rate categories, initiated with 100 parsimony trees and bootstrapped with 1,000 replicates. Bayesian Inference phylogenies for both families were created using MrBayes 7.2.7 on the Cipres Science Gateway 3.3 (Miller et al. 2010). The searches used a mixed amino acid model and consisted of two parallel chain sets run at default temperatures with a sample frequency of 100. The analysis consisted of 5,000,000 generations, with a burnin of 125,000, before calculating posterior probabilities.

To thoroughly test the phylogenetic separation of *Codosiga* and *Hartaetosiga*, respectively *Monosiga* and *Mylnosiga*, an additional ML analysis was done constraining all species of the former genus *Codosiga*, e.g. *Monosiga*, to be monophyletic (matching the morphological base nomenclature) as implemented in RaxML (Stamatakis 2006). Both trees, the constrained and unconstrained, were compared applying the one-side SH test (Shimodaira and Hasegawa 1999) and approximately unbiased (AU) test implemented in CONSEL (Shimodaira and Hasegawa 2001). The one-sided version of the test was used because the gene tree is the ML tree and therefore we expected its likelihood to always be higher or equal than the likelihood associated to the constrained species tree.

Nomenclatural acts

The electronic edition of this article conforms to the requirements of the amended International Code of Zoological Nomenclature, and hence the new names contained herein are available under that Code from the electronic edition of this article. This published work and the nomenclatural acts it contains have been registered in ZooBank, the online registration system for the ICZN. The ZooBank LSIDs (Life Science Identifiers) can be resolved and the associated information viewed through any standard web browser by appending the LSID to the prefix "http://zoobank.org/". The LSID for this publication is: urn:lsid:zoobank.org:pub. The electronic edition of this

work was published in a journal with an ISSN, and has been archived and is available from the following digital repositories: PubMed Central, LOCKSS.

Results

Species misidentification and taxonomic revisions within craspedida

Nitsche et al. (2011) highlighted 23 choanoflagellate species misidentifications both within culture collections and DNA databases; however no attempt was made in that work to revise choanoflagellate taxonomy at the species or generic level. In addition to describing three new craspedid genera and five new species (Fig. 1, Table 1 and Taxonomic Summary), we take the opportunity here to clarify the taxonomic descriptors of a further ***eleven*** craspedid species (Table 1 and Taxonomic Summary).

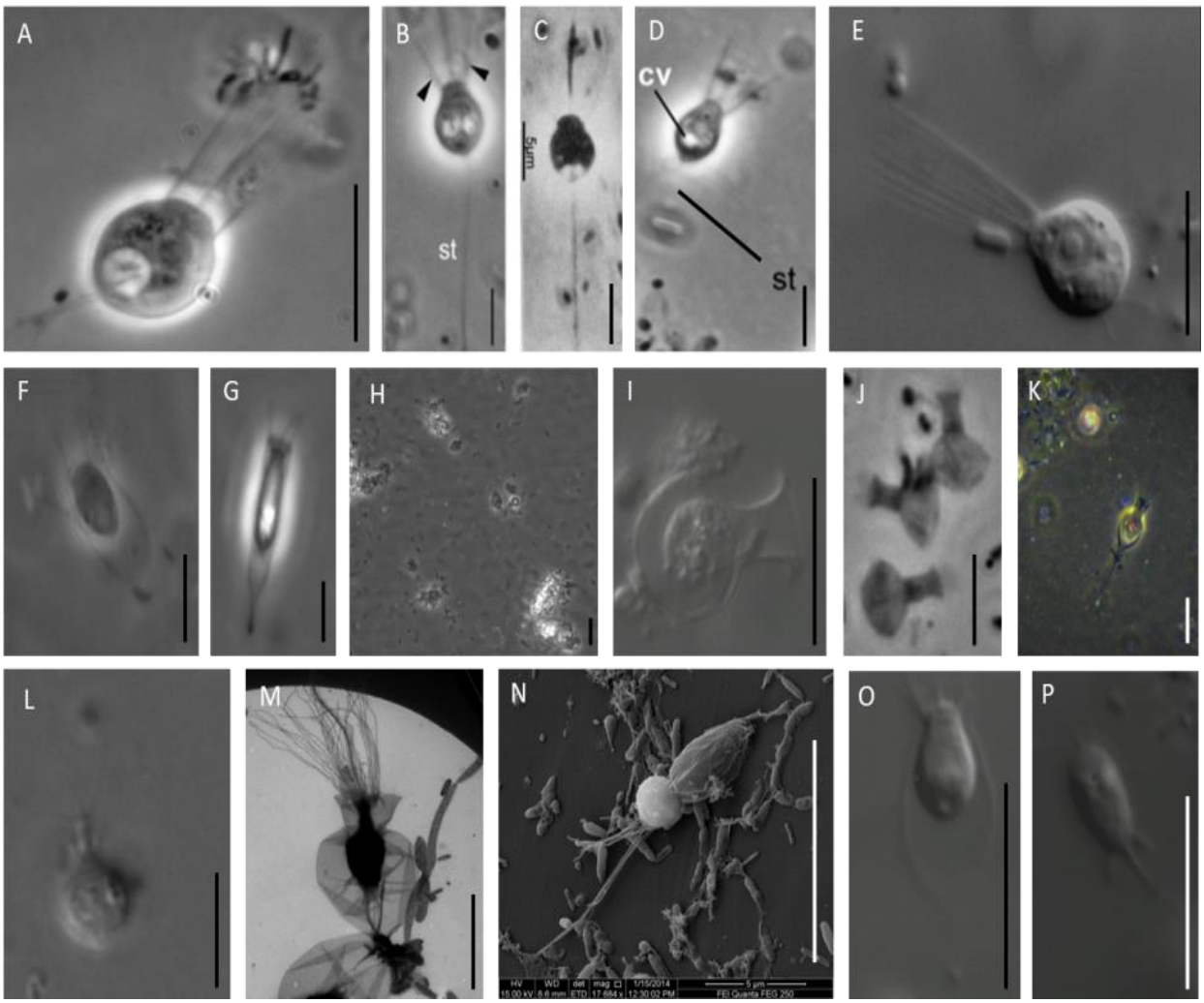

Figure 1. Species described in this study. (A) *Codosiga hollandica*, (B) *Hartaetosiga balthica*, (C) *Hartaetosiga gracilis*, (D) *Hartaetosiga minima*, (E) *Mylnosiga fluctuans*, (F) *Salpingoeca calixa*, (G) *Salpingoeca dolichotheca*, (H) *Salpingoeca helianthica*, (I) *Salpingoeca limnea* (J) *Salpingoeca macrocollata*, (K) *Salpingoeca ohaui*, (L) *Salpingoeca punica*, (M) *Salpingoeca qvevrii*, (N) *Salpingoeca roanoka*, (O) *Stagondion pyriformis* (sedentary cell), (P) *Stagondion pyriformis* (swarmer cell). All scale bars indicate 10 µm.

Table 1. Taxa described in this study.

Species Name	ATCC/NCBI Identifier	Previous Identifier
Codosiga hollandica Carr, Richter and Nitsche	ATCC PRA-391/	Newly described species
Hartaetosiga balthica Carr, Richter and Nitsche	ATCC 50964/1194294	Codosiga balthica Wylezich et Karpov; *Monosiga gracilis*
Hartaetosiga cymosa (Saville Kent) Carr, Richter and Nitsche	-/-	*Codosiga cymosa* Saville Kent
Hartaetosiga gracilis (Saville Kent) Carr, Richter and Nitsche	ATCC 50454/216892	*Codosiga gracilis* Saville Kent
Hartaetosiga minima (Wylezich et Karpov) Carr, Richter and Nitsche	-/1194293	*Codosiga minima* Wylezich et Karpov
Mylnosiga fluctuans Carr, Richter and Nitsche	ATCC 50635/81526	*Monosiga ovata*
Salpingoeca calixa Carr, Richter and Nitsche	-/	Newly described species
Salpingoeca dolichothecata Carr, Richter and Nitsche	ATCC 50959/	*Salpingoeca gracilis*
Salpingoeca helianthica Carr, Richter and Nitsche	ATCC 50153/ 1042118	*Salpingoeca napiformis*

Salpingoeca limnea Carr, Richter and Nitsche	-/	Newly described species
Salpingoeca macrocollata Carr, Richter and Nitsche	ATCC 50938/ 1009460	*Salpingoeca minuta*
Salpingoeca oahui Carr, Richter and Nitsche		Newly described species
Salpingoeca punica Carr, Richter and Nitsche	ATCC 50788/ 1042119	*Salpingoeca amphoridium*
Salpingoeca qvevrii Carr, Richter and Nitsche	ATCC 50929/473812	*Salpingoeca pyxidium*
Salpingoeca roanoka Carr, Richter and Nitsche	ATCC 50931/ 1051735	*Salpingoeca* sp.
Stagondion pyriformis Carr, Richter and Nitsche		Newly described species

Phylogenetic analyses of 42 choanoflagellate species

The newly generated gene sequences were incorporated into a six-gene phylogenetic framework, in an alignment with sequences from 52 holozoan taxa (of which 42 were choanoflagellates). The resulting phylogeny is shown in Fig. 2. Consistent with previous studies, the choanoflagellates were recovered as monophyletic with strong support (99% maximum likelihood bootstrap percentage (mlBP) and 1.00 Bayesian inference posterior probability (biPP)), as was both Craspedida (92% mlBP, 1.00 biPP) and Acanthoecida (100% mlBP, 1.00 biPP).

The nine species previously attributed to *Codosiga* are found in two distinct positions within Craspedida. The marine *C. balthica*, *C. gracilis* and *C. minima* are found in a group which corresponds to Clade 1 of Carr et al. (2008) and form a monophyletic group with strong support (100%mlBP, 1.00biPP). In contrast to the two different and unsupported positions recovered by Stoupin et al. (2012) and Paps et al. (2013), the freshwater *Codosiga* species form a monophyletic group (100%mlBP, 1.00biPP) nested deeply within the Clade 2 choanoflagellates. The two groups of *Codosiga* taxa are shown to be distant relatives of each other and are separated from each other by nine branches in the phylogeny. The genus *Codosiga* is therefore clearly not recovered as monophyletic, with the polyphyly of the genus being a more parsimonious explanation than *Codosiga* paraphyly (2 unweighted parsimony steps rather than 8 unweighted parsimony steps).

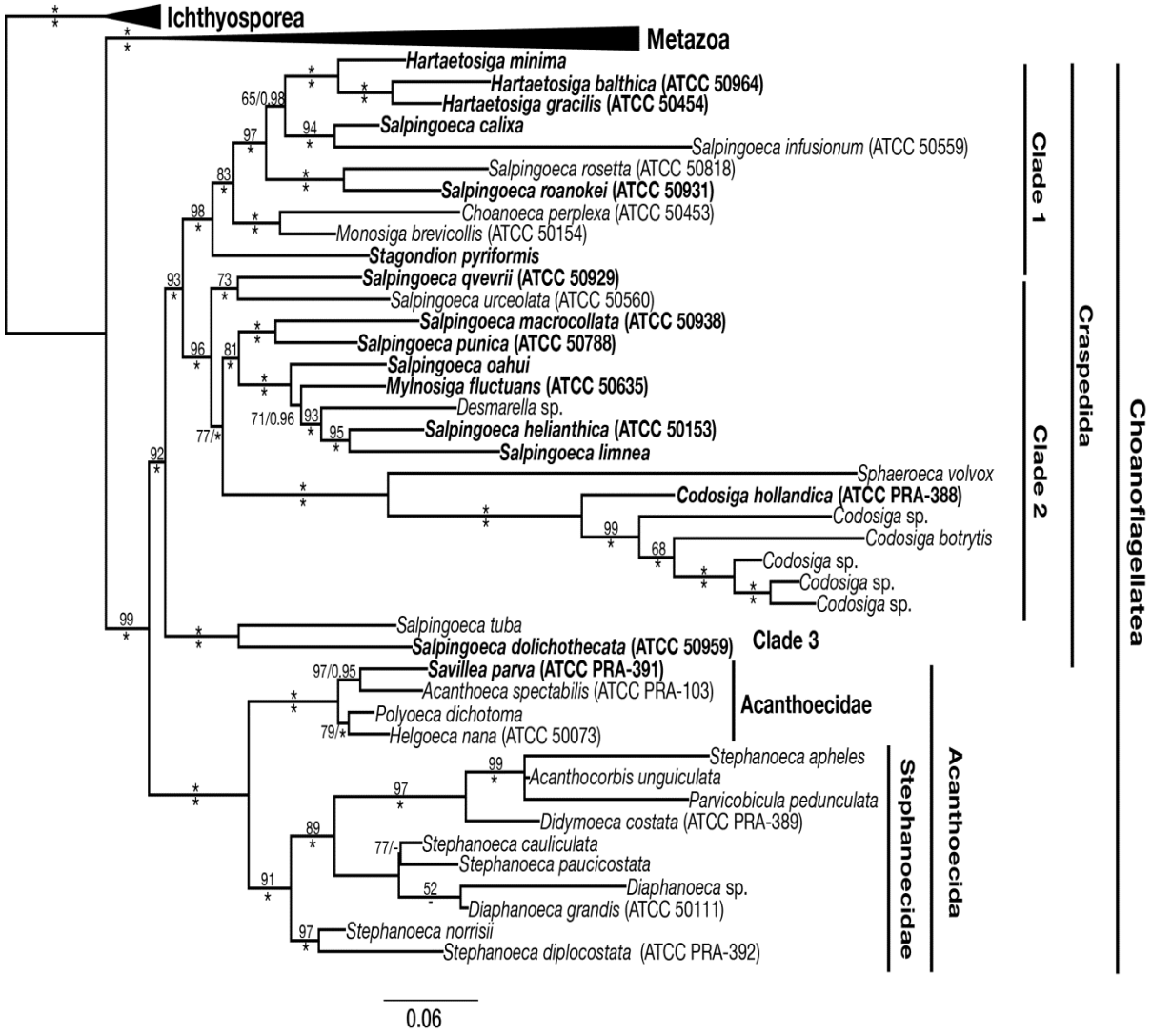

Figure 2. Maximum likelihood six-gene phylogeny of the choanoflagellates. The phylogeny is based upon 9463 aligned nucleotides positions from partial sequences of the genes SSU, LSU, tubA, hsp90, EFL and EF-1A. Branches are drawn proportional to the number of nucleotide substitutions per site as indicated by the scale bar at the lower left. Values of 1.00 biPP and 100% mlBP support are denoted by an *****, mlBP and biPP values are otherwise given above and below branches respectively. Values are omitted from weakly supported branches (i.e. mlBP<50% and biPP<0.70). Species described here are written in bold font.

The type species, *C. botrytis*, is a member of the freshwater clade and accordingly the freshwater species retain the generic name. The marine taxa should no longer be considered as members of *Codosiga* and we therefore have erected a new genus, *Hartaetosiga*, to accommodate them (see Taxonomic Diagnoses).

A marine thecate species from Bálos Lagoon was found to have an ovoid thecate morphology distinct from the familiar cup, flask and tube forms (see Fig. 1). The ovoid theca has a narrow anterior aperture from which the collar and flagellum extend, however there is no narrow neck as is observed in the flask bearing species. At the posterior pole, the theca tapers into a short peduncle. Due to the theca having a droplet-like shape this species has been placed into a new genus, *Stagondion*, as the holotype with the name *S. pyriformis* (Taxonomic Diagnoses). *S. pyriformis* is recovered at the base of the Clade 1 craspedids with strong support (98%mlBP, 1.00biPP).

S. tuba and ATCC 50959 are the first tube thecate species included in a multi-gene framework. ATCC 50959 was previously shown to have been misidentified (Nitsche et al. 2011) and we describe it here under the name *Salpingoeca dolichothecata* (see Fig. 1, Taxonomic Diagnoses). The two tube thecate species form a robust grouping (100%mlBP, 1.00biPP) and are recovered at the base of Craspedida as a sister-group to the other craspedids with strong support (92%mlBP, 1.00biPP).

Partial fragments of both SSU and LSU were sequenced from two novel flask species. The species were isolated from O'ahu, Hawaii and Greenland and have been named as *Salpingoeca oahui* and *S. limnea* respectively (see Fig. 1, Taxonomic Diagnoses). Both species fall into a large paraphyletic group of freshwater species, which is recovered with strong support in our phylogeny (77%mlBP, 1.00biPP). Furthermore a novel cup thecate from McKenzie Bay, New Zealand, described under the name *S. calixa* (see Fig. 1, Taxonomic Diagnoses), clusters in a paraphyletic group comprising cup thecate species (97%mlBP, 1.00biPP). Nested within the cups are the three *Hartaetosiga* species.

Finally, an undescribed freshwater, naked, craspedid, isolated from the Atlantic island of Madeira, is recovered with strong support (100%mlBP, 1.00biPP) with the freshwater *Codosiga* species. The species has been described as *C. hollandica* (see Fig. 1, Taxonomic Diagnoses).

Both tests, SH and AU, of the constraint trees compared to the unconstraint one showed a highly significant p-value in favor of the phylogenetic based tree, hence favoring a non-monophyletic origin of the two examined genera. The p-value for the unconstraint tree was 1.00 for both tests compared to 2e-007 (AU) and 0.00 (SH) for the tree constraint to a monophyletic origin of the genus *Codosiga*, e.g. 1e-076 (AU) and 0.00 (SH) for the tree constraint to a monophyletic origin of the genus *Monosiga*.

Evolutionary trends within the choanoflagellates

The phylogeny presented here places 42 choanoflagellate species into a phylogenetic context and therefore provides an unprecedented opportunity to evaluate the evolution of morphological, ecological and genomic traits within the group.
A matrix of characters for each species in the phylogeny is available from the author upon request. Nine of the major traits listed in the matrix are discussed in greater detail below.

Flask-theca morphology

The flask-theca is perhaps the most structurally complex of the known thecal morphologies. This is because, apart from its precise shape, it possesses a flange on the inner surface of the neck which attaches to the anterior end of the cell. On the outer surface, the neck is often decorated with a pattern of narrow ridges (Fig. 3). It is the only thecal morphology that is present in both clades 1 and 2 of Craspedida within the phylogeny. One possible explanation for the distribution of the flask-thecates is that the flask was the ancestral thecal morphology of both clades 1 and 2

craspedids. An alternative scenario, of convergent evolution, seems unlikely due to the remarkable similarity of the complex morphology observed in both Clade 1 and Clade 2 flask-thecates (Fig. 3).

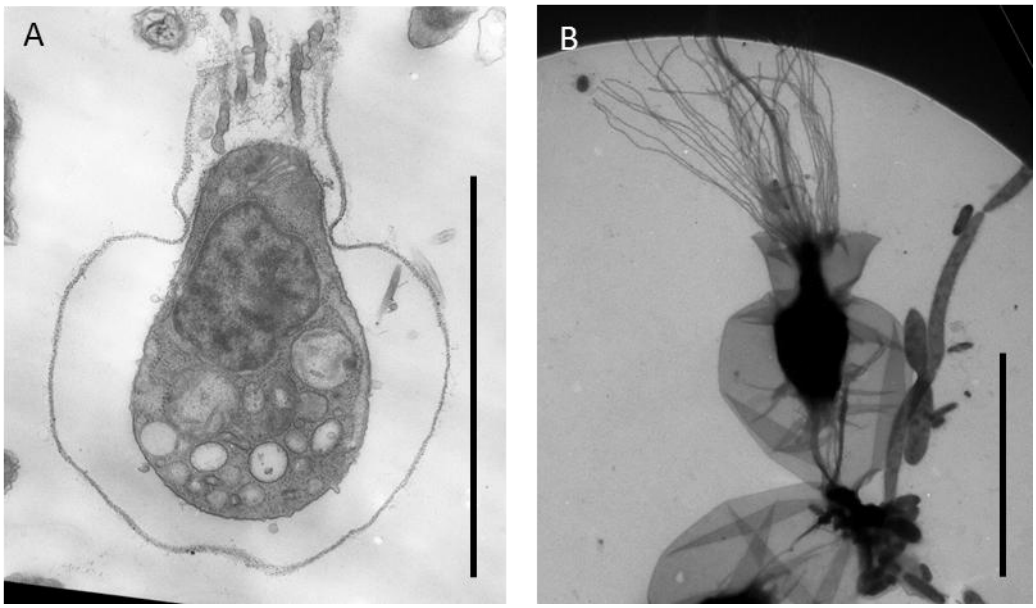

Figure 3. Morphological similarities of the flask theca between (A) *Choanoeca perplexa* (Clade 1) and (B) *Salpingoeca qvevrii* (Clade 2). All scale bars indicate 10 µm.

Tube-theca morphology

S. dolichothecata and *S. tuba* are the first species with tube thecae to be placed in a multi-gene phylogenetic framework and provide insights into the origin of the tube morphology. The two species form a strongly supported monophyletic group (100%mlBP, 1.00biPP), consistent with a single origin of the tube morphology. The tube species are recovered within the craspedids (92%mlBP, 1.00biPP) with strong support, as the earliest branching lineage, and form a third, previously unidentified clade, within this taxon (Fig. 2).

Cup-theca morphology

All four species in the phylogeny that possess the cup-theca morphology cluster together with strong support (97%mlBP, 1.00biPP) in Clade 1 of the craspedids. Furthermore, a fifth cup-thecate, *Salpingoeca abyssalis*, has previously been shown to group with the species shown here (Nitsche et al. 2011). The cup grouping is however paraphyletic, with moderate to strong phylogenetic support (65%mlBP, 0.98biPP), as the three *Hartaetosiga* species are recovered as being nested within it. The phylogeny is consistent with a putative single origin for the cup morphology; however multiple origins of the cup morphology in closely related taxa cannot be discounted. If the scenario of a single origin of the cup is correct, then the naked *Hartaetosiga* appear to have evolved from a cup-bearing ancestor.

Lorica morphology

The basket-like lorica, containing costae made up of silica costal strips, is a highly distinctive cell covering and unique to the Acanthoecida. Based upon a sample of six species, Carr et al. (2008) recorded morphological differences between the Acanthoecidae and Stephanoecidae families, respectively termed nudiform and tectiform species, and these distinctions are confirmed in the 14 species in this study. Longitudinal costae are present in all loricate species in the phylogeny, with the exception of *Acanthoeca spectabilis*, suggesting that they evolved in a stem-group loricate. Loricae with rings are unique and universal to the stephanoecids, indicating that the morphology evolved in a stem-group stephanoecid subsequent to divergence of the acanthoecid and stephanoecid lineages. The other form of lateral strips, helical costae, is universal in the known acanthoecid species. Furthermore helical costae are also broadly distributed within the stephanoecids, thus it is probable that the LCA of the loricates had a lorica composed from both helical and longitudinal costae.

Number of cells per peduncle

It is clear that there is no phylogenetic dichotomy between species with a single cell on a peduncle (the monosigid morphology) and those with multiple cells on a peduncle (the codosigid morphology). As the ancestral cell covering of the craspedids appears to have been a theca (Nitsche et al. 2011), the 'naked' appearances of the *Codosiga* and *Monosiga* morphologies are almost certainly derived states. The current phylogeny indicates that both the codosigid and monosigid morphologies have evolved on at least two occasions, with both *Codosiga* and *Monosiga* being recovered as polyphyletic (Taxonomic Summary). Furthermore, taxa with the capacity to develop multiple cells on a single peduncle may be mistaken as monosigid species when they initially settle onto a surface prior to cell division (Leadbeater and Morton 1974; Wylezich et al. 2012). The number of cells per peduncle therefore appears to be a plastic morphological trait and unreliable for choanoflagellate taxonomy.

Coloniality

The ability of choanoflagellates to form ephemeral colonies has long been recognized (Fromental 1874; Stein 1878) and a possible evolutionary link between coloniality in choanoflagellates and multicellularity in metazoans has previously been speculated upon (Carr et al. 2008; Dayel et al. 2011). Colonies may take the form of (1) chains of cells, (2) free-swimming spheres of cells, (3) plate-like assemblages of cells or (4) multiple sedentary cells attached to a single peduncle (Leadbeater 1983; Carr and Baldauf 2011). Recent work has shown that individual species are capable of developing multiple colonial morphologies (Dayel et al. 2011). This important finding casts further doubt on the reliance of morphological traits in the taxonomy of craspedid choanoflagellates, as colonial forms attributable to *Desmarella*, *Proterospongia* and *Sphaeroeca* have been found in a clonal culture of the same species.

Coloniality has been observed in 17 of the 28 craspedid species present in the current phylogeny. Coloniality cannot be excluded in any of the other craspedids, as most have poorly studied life cycles; however it is unlikely that it is a trait of some species, such as *M. brevicollis* or *Mylnosiga fluctuans* (erroneously deposited at ATCC as *M. ovata*, see Nitsche et al. 2011 and Taxonomic Summary), which have been intensively studied. The common structure of intercellular cytoplasmic bridges present in Clade 1 (*S. rosetta*, see Dayel et al. 2011) and Clade 2 (*Desmarella moniliformis* and *C. botrytis*, see Leadbeater and Karpov 2000, Hibberd 1975) either suggests that such bridges were present early in craspedid evolution, or that there has been a remarkable level of convergent evolution within the group. Similar cytoplasmic bridges are also present between metazoan cells (Fairclough et al. 2013), suggesting such bridges may have much greater antiquity. To date no acanthoecids have been shown to form colonies via connections between protoplasts, however this may be due to the restrictive nature of their siliceous loricae preventing intercellular bridges forming.

Juvenile dispersal stage

The stephanoecids are the only choanoflagellate species that do not have a swimming, naked, juvenile dispersal stage (Leadbeater and Cheng 2010). All other species have the potential to either immediately settle on a surface and develop a peduncle or disperse after cell division via a flagellated cell that possesses a fine extracellular investment. Unweighted parsimony cannot differentiate between a single gain, in a stem-group choanoflagellate, followed by loss of the juvenile dispersal stage in the stephanoecids and two independent gains of the dispersal stage in Craspedida and Acanthoecidae. However the broad distribution of uniflagellated dispersal cells in Opisthokonta (e.g. chytrid fungi, ichthyosporeans, choanoflagellates and metazoan spermatozoa, Carr and Baldauf 2011) suggests that the LCA of the choanoflagellates had a juvenile dispersal stage.

Freshwater-marine transitions

The phylogeny illustrated in Fig. 2 contains 13 freshwater and 29 marine species of choanoflagellate. All of the freshwater taxa fall into a single, paraphyletic group (77%mlBP, 1.00biPP) in the phylogeny. The single marine species in this group, *S. macrocollata,* is robustly nested deep within the freshwater species.

The current phylogeny requires a minimum of two freshwater:marine transitions to explain the distribution of sampled species and the phylogeny is consistent with a single freshwater invasion by the craspedids. Multiple incursions, by closely related species, is a less parsimonious explanation (4 unweighted steps against 3 unweighted steps) for the distribution of freshwater species but cannot be excluded. It is clear however that the freshwater environment has been invaded by choanoflagellates on more than one occasion. In addition to the colonization of freshwater by craspedids highlighted here, Paul (2011) showed an incursion by the acanthoecid *Acanthocorbis mongolica*, present over 2 consecutive years in the freshwater lake Bayan Nuur, Mongolia. Furthermore del Campo and Ruiz-Trillo (2013) performed a meta-analysis of environmental SSU sequences which recovered eleven putative clades containing freshwater choanoflagellates. As with previously published choanoflagellate SSU phylogenies (Carr et al. 2008; Cavalier-Smith and Chao 2003) the deeper branches within the choanoflagellates were poorly resolved and one of the putative freshwater clades (Freshcho3/Clade L) was recovered outside the diversity of known choanoflagellate species. Representative sequences from each of the putative del Campo and Ruiz-Trillo (2013) freshwater clades were placed into our six-gene alignment and phylogenies were created using maximum likelihood and Bayesian inference methods (Supplement Fig. 1). All eleven environmental sequences were recovered within the choanoflagellate clade. Three sequences, GU290082, GU647175 and GU647190, which were isolated from freshwater lakes in Africa and North America, fell with strong support (mlBP≥75%, biPP≥0.97) in the freshwater group recovered in the main phylogeny. The environmental sequences greatly reduce the phylogenetic support across the tree

and only one of the eight remaining environmental sequences has a strongly supported position. This sequence, AM179824, which was isolated from the hypersaline Laguna Tebenquiche, Chile, forms a strongly supported sister-grouping with the marine *S. pyriformis* (80% mlBP, 1.00biPP). The lack of phylogenetic support means that the environmental sequences provide equivocal evidence for multiple incursions into the freshwater environment by choanoflagellates, with five putative groups being recovered.

The available data point to freshwater:marine transitions being rare events in choanoflagellate evolution, as is the case in many protistan groups (Logares et al. 2009).

Distribution of EFL and EF-1A

Transcriptome data from 19 species of choanoflagellate were screened with hidden Markov model profiles of both EF-1A and EFL. Both profiles retrieved identical top hits from each choanoflagellate species, showing that no species possessed both genes. Combining the transcriptome data with publicly available data shows that, of 22 choanoflagellate species, 17 species possess EFL and five species encode EF-1A (Fig. 4, Supplement Fig. 2).

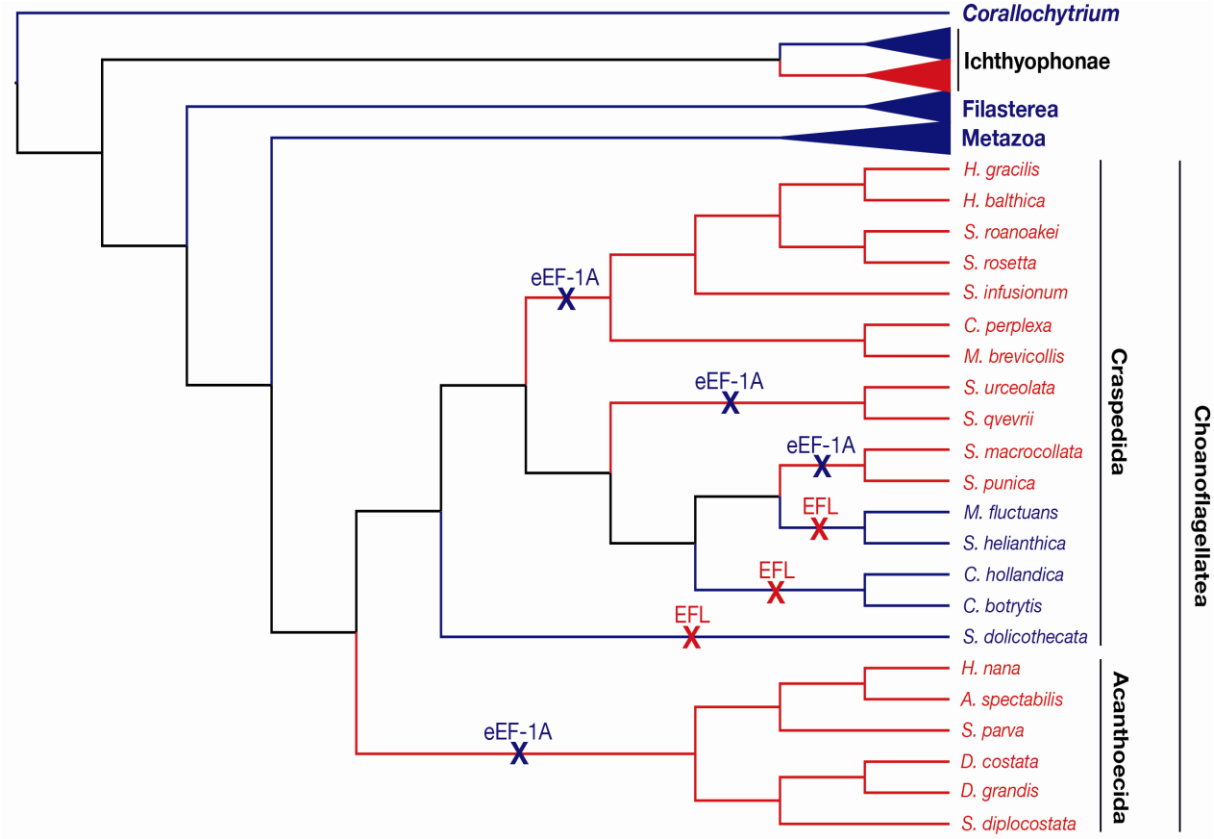

Figure 4. Loss of EFL and EF-1A within the choanoflagellates. The simplified phylogeny is based upon Fig. 2 and Paps et al. (2013). Lineages coloured red encode EFL and blue lineages encode EF-1A. 'X' denotes the loss of a gene.

The maximum likelihood phylogeny of EF-1A within opisthokont species (Supplement Fig. 2) recover the choanoflagellate sequences as a weakly supported monophyletic group (mlBP<50%) nested within a paraphyletic grouping of EF-1A from metazoans and the filasterean *Ministeria vibrans*. The relationship between the metazoan and choanoflagellate EF-1A sequences has no phylogenetic support (all branches <25%mlBP, <0.50biPP). The phylogeny of EF-1A is however broadly consistent with the vertical inheritance of the gene throughout the opisthokont radiation. The choanoflagellate LCA appears to have possessed EF-1A with at least four subsequent, independent losses of the gene within the choanoflagellates (Fig. 4).

An amino acid phylogeny of 133 EFL sequences clustered the choanoflagellate proteins together; the group was recovered as paraphyletic since sequences from three ichthyosporeans (*Creolimax fragrantissima*, *Sphaeroforma arctica* and *S. tapetis*) were nested within it (Supplement Fig. 3). The placement of the ichthyosporean EFL sequences within those of the choanoflagellates is however not recovered with strong phylogenetic support (mlBP<75%, biPP<0.97).

The topology of the choanoflagellate EFL sequences is consistent with the six-gene phylogeny shown in Fig. 2, indicating that the gene was present in the choanoflagellate LCA and has been inherited vertically during the choanoflagellate radiation. Mapping the presence and absence of EFL onto the choanoflagellate phylogeny shows a minimum of three independent losses within the choanoflagellates, all of which have occurred within Craspedida (Fig. 4). The holozoan EFL sequences are recovered together with strong support (77%mlBP, 1.00biPP) and the phylogeny is consistent with the presence of the gene in the LCA of choanoflagellates and ichthyosporeans. The phylogeny does not however cluster the holozoan sequences with those of the fungi as might be expected if EFL had undergone vertical inheritance throughout opisthokont evolution, however it must be stressed that there are no strongly supported branches in the backbone of the phylogeny between the fungal and holozoan EFL sequences. The holozoan EFLs do not cluster strongly with any other group, but form a poorly supported cluster with sequences from rhodophytes, two plants and a cryptophyte (Supplement Fig. 3).

Discussion

In addition to the considerable phylogenetic distance between *Codosiga* and *Hartaetosiga*, species from the two genera also show numerous morphological and ecological differences further justifying their separation. In particular, the cell covering of *Codosiga* has two appressed layers and has been shown to be more substantial than that of the single-layered investment of *Hartaetosiga* (Leadbeater and Morton 1974; Hibberd 1975; Wylezich et al. 2012). Leadbeater (1977) noted that the single layered cell coat of *H. gracilis* is similar to that observed in motile cells of *Choanoeca perplexa* Ellis, which is also a Clade 1 craspedid (Fig. 2). Furthermore, the cell body extends into the periplast stalk in *Codosiga*, but does not in *Hartaetosiga*.

Within the cell, the microtubules around the flagellar base are symmetrical in *H. gracilis* (Leadbeater and Morton 1974), but asymmetrical in *C. botrytis* (Hibberd 1975). In multi-headed colonies, the cells of *C. botrytis* do not fully undergo cytokinesis and are connected by cytoplasmic bridges (Hibberd 1975); however such bridges have not been reported in the multi-headed colonies of *Hartaetosiga* species.

The newly described *Hartaetosiga* genus is nested within the grouping of cup-thecate species with moderate to strong support (65% mlBP, 0.98 biPP), which suggests that the naked *Hartaetosiga* species may have evolved from a cup-bearing ancestor. The amended *Codosiga* genus is recovered as a close relative of *Sphaeroeca* (represented by the type species, *Sphaeroeca volvox* Lauterborn) with strong support (100% mlBP, 1.00 biPP). *Sphaeroeca* is composed exclusively of naked craspedid cells and four of the five recognized *Sphaeroeca* taxa (*S. globosa*, *S. lackeyi*, *S. pedicellata* and *S. volvox*) are freshwater species (Leadbeater 1983). The available data therefore indicate that the LCA of *Sphaeroeca* and *Codosiga* was a freshwater, naked choanoflagellate that was capable of forming colonies.

We have begun to establish a degree of order within the taxonomy of the craspedids by splitting taxa previously assigned to *Codosiga* into two phylogenetically, morphologically and ecologically coherent genera. Two further new genera,

Stagondion and *Mylnosiga*, have been erected in order to accommodate ovoid thecate species and a naked, free-swimming craspedid. Furthermore, the taxonomy of ***9*** misidentified species present in culture collections has been resolved. Obvious problems within craspedid taxonomy remain with the paraphyletic *Salpingoeca*, the latter of which can be considered a 'dustbin' genus that shows no rationale on a phylogenetic, ecological or morphological level. Unfortunately, DNA sequences are not available for the type species, *Salpingoeca gracilis*, which has not been deposited in a culture centre. *S. gracilis*, is a tube thecate species, therefore it would be preferable, when possible, for future taxonomic revisions to assign non-tube thecate species to other genera. Within the Stephanoecids, the paraphyletic *Stephanoeca* also appears to require considerable revision (Fig. 2). However, phylogenetic support is weak for many branches in the Stephanoecids and sequence data are unavailable for the type species *S. ampulla*.

The plastic nature of cell coverings, as well as the ability of species to develop multiple morphologies presents practical problems for choanoflagellate taxonomy. Future work may require a consensus within the choanoflagellate community whether to take a 'lumper' or 'splitter' approach to the taxonomy of the group. The lumper approach would result in genera with common, but not universal, morphological traits; a splitter approach however is likely to result in a myriad of small, but morphologically coherent, genera.

The revised six-gene phylogeny presented here greatly increases the number and diversity of named choanoflagellate species placed into a phylogenetic framework. Importantly the tube and ovoid thecate species are added to the phylogeny and give insights into their origins. The phylogeny is consistent with a single origin of the cup, flask and tube thecate taxa, however multiple origins of the cups and flasks cannot be discounted.

The phylogeny highlights the presence of a major freshwater radiation in the Clade 2 craspedids, however environmental sequencing of SSU raises the possibility of greater freshwater diversity and additional freshwater incursions. This data should be treated with a degree of caution, as, with the exception of a sequence from the

hypersaline Laguna Tebenquiche, the environmental sequences do not have strongly supported phylogenetic relationships with marine choanoflagellates.

Finally, transcriptome data provide strong evidence that the choanoflagellate LCA possessed both EF-1A and EFL in its genome. Both genes have subsequently been lost on multiple occasions, with more studied species possessing EFL rather than EF-1A. As with smaller scale EFL phylogenies (Noble et al. 2007; Kamikawa et al. 2010a,b), the deeper branches are poorly resolved meaning that it is currently difficult to speculate on the evolutionary origin of EFL within the holozoans. The EFL phylogeny is consistent with the gene being present, along with EF-1A, in the LCA of the choanoflagellates and ichthyosporeans. If this scenario is correct, then both genes were also present in the LCA of choanoflagellates and metazoans, with EFL apparently being lost in a stem-group metazoan. These data highlight a major difference in metabolism between choanoflagellates and metazoans, as EF-1A is universal in metazoan protein synthesis (Kamikawa et al. 2010b) whilst the majority of choanoflagellates employ EFL.

Taxonomic summary

Codosiga (James-Clark) emend. Carr, Richter and Nitsche

Type species: *Codosiga botrytis* (Ehrenberg) emend. Leadbeater

Small, uninucleate protists with a single, centrally positioned, anterior flagellum, which is surrounded by a collar of long, actin-supported microvilli. Phagotrophic. Sedentary cells have a posterior, two layered, extracellular investment that extends into a peduncle. The protoplast extends posteriorly into the peduncle. Mature cells do not produce a rigid theca. Stalked mature cells can divide to form multi-headed colonies. Colonial cells may be connected by means of cytoplasmic bridges. Mitochondrial cristae flattened. All known species are found in freshwater.

***Codosiga hollandica* sp. nov. Carr, Richter and Nitsche**

Protoplast almost spherical, with a slight pinched waist below the collar. Peduncle shorter than diameter of protoplast.

Etymology: The SSU sequence for this species is essentially identical to a number of uncultured eukaryotic sequence clones found at a water treatment plant in the Netherlands (roughly 30 clones with the identifier BSF-B-15euk; Valster et al., 2010). The species is named after Holland, a common colloquial name used as a *pars pro toto* to refer to the Netherlands.

***Hartaetosiga* gen. nov. Carr, Richter and Nitsche**

Type species: *Hartaetosiga* (*Codosiga*) *gracilis* (Saville Kent) Carr, Richter and Nitsche

Small, uninucleate protists with a single, centrally positioned, anterior flagellum, which is surrounded by a collar of long, actin-supported microvilli. Phagotrophic. Cell body possesses a distinctive waist behind the attachment of the collar microvilli. Posterior region of cell body enclosed in a delicate single-layered organic investment from which a peduncle extrudes. The cell body does not extend into the peduncle. Stalked mature cells can divide to form multi-headed colonies of cells. Mitochondrial cristae flat or tubular. All known species are marine.

Etymology: The name is derived from the Latin *Hartaetus* meaning kite, since the appearance and movement of cells on long stalks is reminiscent of kites flying on lines.

***Hartaetosiga* (*Codosiga*) *balthica* (Wylezich et Karpov) Carr, Richter and Nitsche**

Cell body is 3-4.5µm in length and 2µm in width. Collar is 3-4.5µm in length. Adult sedentary protoplast present in delicate extracellular investment which produces a peduncle 9-14 µm in length. Protoplast globular to pyriform in shape. Mitochondrial cristae tubular.

Note. This species was deposited at ATCC under the name *Monosiga gracilis*. *M. gracilis*, as originally described by Saville Kent (1880-1882), only produced a single cell per peduncle, whereas *H. balthica* can produce 2-4 cells per peduncle. Based upon the morphological differences *H. balthica* and *M. gracilis* appear to be different species.

Subjective synonym: ATCC 50964

***Hartaetosiga* (*Codosiga*) *cymosa* (Saville Kent) Carr, Richter and Nitsche**

syn. Codosiga cymosa Saville Kent 1880

Cell body symmetrically ovate. Single cell per individual peduncle in sedentary adult cells. Peduncles arranged on an elaborate corymbiform pedicel which may be composed of over of 100 individuals.

***Hartaetosiga* (*Codosiga*) *gracilis* (Saville Kent) Carr, Richter and Nitsche**

syn. Monosiga gracilis Saville Kent 1880, *Codosiga pyriformis* Saville Kent, *Codosiga gracilis* (Saville Kent 1880) Saedeleer 1927

Cell body is 4-8µm in length and 3-7µm in width. Collar is 8-20µm in length. Sedentary mature cells produce a peduncle of 8-40µm in length. Cell body tapers towards, but does not extend into, the peduncle.

Subjective synonym: ATCC 50454

***Hartaetosiga* (*Codosiga*) *minima* (Wylezich et Karpov) Carr, Richter and Nitsche**

Cell body is 2-3µm in length and 1µm in width. Collar is 2-3µm in length. Adult sedentary protoplast present in delicate extracellular investment that extends into a peduncle 7µm in length. Protoplast pyriform in shape. Mitochondrial cristae oval.

M*ylnosiga* gen. nov. Carr, Richter and Nitsche

Type species: *Mylnosiga fluctuans* Carr, Richter and Nitsche

Spherical protoplast present in a delicate extracellular investment, which does not extend to a pedicel. Mature cells are not sessile, but drift in water currents. Long collar and flagellum. Freshwater.

***Mylnosiga fluctuans* sp. nov. Carr, Richter and Nitsche**

Spherical protoplast present in a delicate extracellular investment, which does not extend to a pedicel. Long collar and flagellum, extending more than twice the body length. Freshwater.

Note. This species was deposited at ATCC in 1979 under the name *Monosiga ovata*. *M. ovata*, as originally described by Saville Kent (1880-82), is a marine organism which possesses a short peduncle, whereas *M. fluctuans* was isolated from a freshwater pond and does not produce a peduncle. Based upon the morphological and ecological differences ATCC 50635 and *M. ovata* appear to be different species.

Etymology: The name is derived from the Latin *fluctuans* (= floating), as the species is freely suspended in the water column.

Subjective synonym: ATCC 50635

***Salpingoeca calixa* sp. nov. Carr, Richter and Nitsche**

Ovoid adult sedentary cells possess a typical cup-shaped theca, 4.5-7.5µm in length and 2-4µm in width from which the collar and flagellum of the protoplast emerges. Flagellum longer than the collar. Theca tapers gradually into a pedicel of 19-27µm in length. Marine.

Etymology: The name is derived from the Latin *calix*, which refers to the cup morphology of the theca in sedentary cells.

***Salpingoeca dolichothecata* sp. nov. Carr, Richter and Nitsche**

Highly elongated protoplast of sedentary cells contained in a long, straight sided, tubular theca. Collar and flagellum extend above the theca. Base of theca tapers into a short pedicel. Marine.

Note. This species was deposited at ATCC under the name *Salpingoeca gracilis*. *S. gracilis*, as originally described by James-Clark (1867), is a freshwater organism which possesses a long pedicel, whereas *S. dolichothecata* is a marine organism and produces a short peduncle. Based upon the morphological and ecological differences ATCC 50959 and *S. gracilis* appear to be different species.

Etymology: The name is derived from the Greek *dolicho* (= long), which refers to the extended theca of sedentary cells.

Subjective synonym: ATCC 50959

***Salpingoeca helianthica* sp. nov. Carr, Richter and Nitsche**

Adult, sedentary cells present in flask-theca with short, broad neck. Theca extends into a short pedicel. Height of theca, excluding peduncle, greater than the diameter. Freshwater.

Note. This species was deposited at ATCC under the name *Salpingoeca napiformis*. *S. napiformis*, as originally described by Saville Kent (1880), is a marine organism, whereas *S. helianthica* was isolated from a freshwater aquarium. Based upon the ecological difference ATCC 50153 and *S. helianthica* appear to be different species.

Etymology: The name is from the Latin *helianthus*, for sunflower, since the colonial life stage resembles a sunflower, with a dark circular centre surrounded by radially symmetrical cell bodies forming the colony.

Subjective synonym: ATCC 50153

***Salpingoeca limnea* sp. nov. Carr, Richter and Nitsche**

Adult sedentary cells possess a typical flask-theca, 7.5-10µm in length and 7.8-10.9µm in width from which the collar (9.2-12.1µm in length) and flagellum of the protoplast emerges. Theca (length: 12.3-13.1µm; width: 9.2-12.1µm) possesses a short and broad neck with a flared opening. Motile cells can form chain colonies. Freshwater.

Etymology: The name is derived from the Greek –*limne* which refers to the freshwater pond from which this species was isolated.

***Salpingoeca macrocollata* sp. nov. Carr, Richter and Nitsche**

Globular, sedentary, adult cells present in flask-morphology theca with long straight neck. Theca neck height greater than diameter of main body. Marine.

Note. This species was deposited at ATCC in 2001 under the name *Salpingoeca minuta*. *S. minuta*, as originally described by Saville Kent (1880), is a freshwater organism with a short, broad neck within its theca. In contrast, *S. macrocollata* was isolated from a saltmarsh and possesses a long, narrow neck. Based upon the ecological and morphological differences ATCC 50938 and *S. macrocollata* appear to be different species.

Etymology: The name is derived from the Greek *macro*- and Latin -*colla* which refers to the long neck of the theca in sedentary cells.

Subjective synonym: ATCC 50938

S. oahui sp. nov. Carr, Richter and Nitsche

Adult sedentary cells possess a typical flask-theca, 11.5-14.5µm in length and 4.5-6µm in width from which the collar and flagellum of the protoplast emerges. Flagellum considerably longer than the collar. Theca opening at the top of a short and broad neck. Theca tapers gradually into a pedicel of 19-27µm in length. Freshwater.

Etymology: The species is named after the island of O'ahu, where the species was first identified.

***Salpingoeca punica* sp. nov. Carr, Richter and Nitsche**

Adult sedentary cells possess a globular flask-theca, from which the collar and flagellum of the protoplast emerges. Theca opening at the top of a very short, broad neck. Freshwater.

Note. This species was deposited at ATCC under the name *Salpingoeca amphoridium*. *S. amphoridium*, as originally described by Saville Kent (1880), possessed a theca with a long, narrow neck. In contrast, the theca of *S. punica* produces a short, broad neck. Based upon the morphological difference ATCC 50788 and *S. punica* appear to be different species.

Etymology: The theca morphology resembles the shape of pomegranate and the name is derived from the Latin *Punica*, which is the genus name of the pomegranate.

Subjective synonym: ATCC 50788

***Salpingoeca qvevrii* sp. nov. Carr, Richter and Nitsche**

Adult sedentary cells possess a flask shaped theca with short neck. Base of theca rounded with no pedicel. Marine.

Note. This species was deposited at ATCC in 1999 under the name *Salpingoeca pyxidium*. *S. pyxidium*, as originally described by Saville Kent (1880), is a freshwater species, whereas *S. qvevrii* was isolated from a salt marsh. Based upon the ecological difference ATCC 50929 and *S. pyxidium* appear to be different species.

Etymology: The name is taken due to the similarity in shape between the theca of sedentary cells and qvevri wine jars.

Subjective synonym: ATCC 50929

***Salpingoeca roanoka* sp. nov. Carr, Richter and Nitsche**

Etymology: This species is named after the failed English colony of Roanoke, due both to its ability to form transient colonies and its geographical location. The original settlement of Roanoke was established on what later became the Virginia Colony, which encompassed the current US state of Virginia (the collection site of the environmental sample from which the culture was isolated).

Subjective synonym: ATCC 50931

***Stagondion* gen. nov. Carr, Richter and Nitsche**

Type species: *Stagondion pyriformis* Carr, Richter and Nitsche

Small, uninucleate protists with a single, centrally positioned, anterior flagellum, which is surrounded by collar of long, actin-supported microvilli. Phagotrophic. Cell body enclosed in a robust organic theca from which a pedicel extrudes. The theca has an ovoid, or droplet, shaped morphology without a neck.

Etymology: The name is derived from the Greek Stagondion (= droplet), since the thecae of mature cells develops a droplet-shaped morphology.

***Stagondion pyriformis* sp. nov. Carr, Richter and Nitsche**

Ovoid cell body is 3-5µm in length and 3-4.5µm in width. Collar is 8-12µm in length and surrounds a flagellum of ~9µm in length. Sedentary cells produce a pyriform theca 9-11µm in length and 5-6.5µm in width which tapers into a short peduncle. Cell body tapers toward, but does not extend into, the pedicel.

Etymology: The name is taken from the Latin *pyriformis,* which refers to the pear-like morphology of the theca in sedentary cells.

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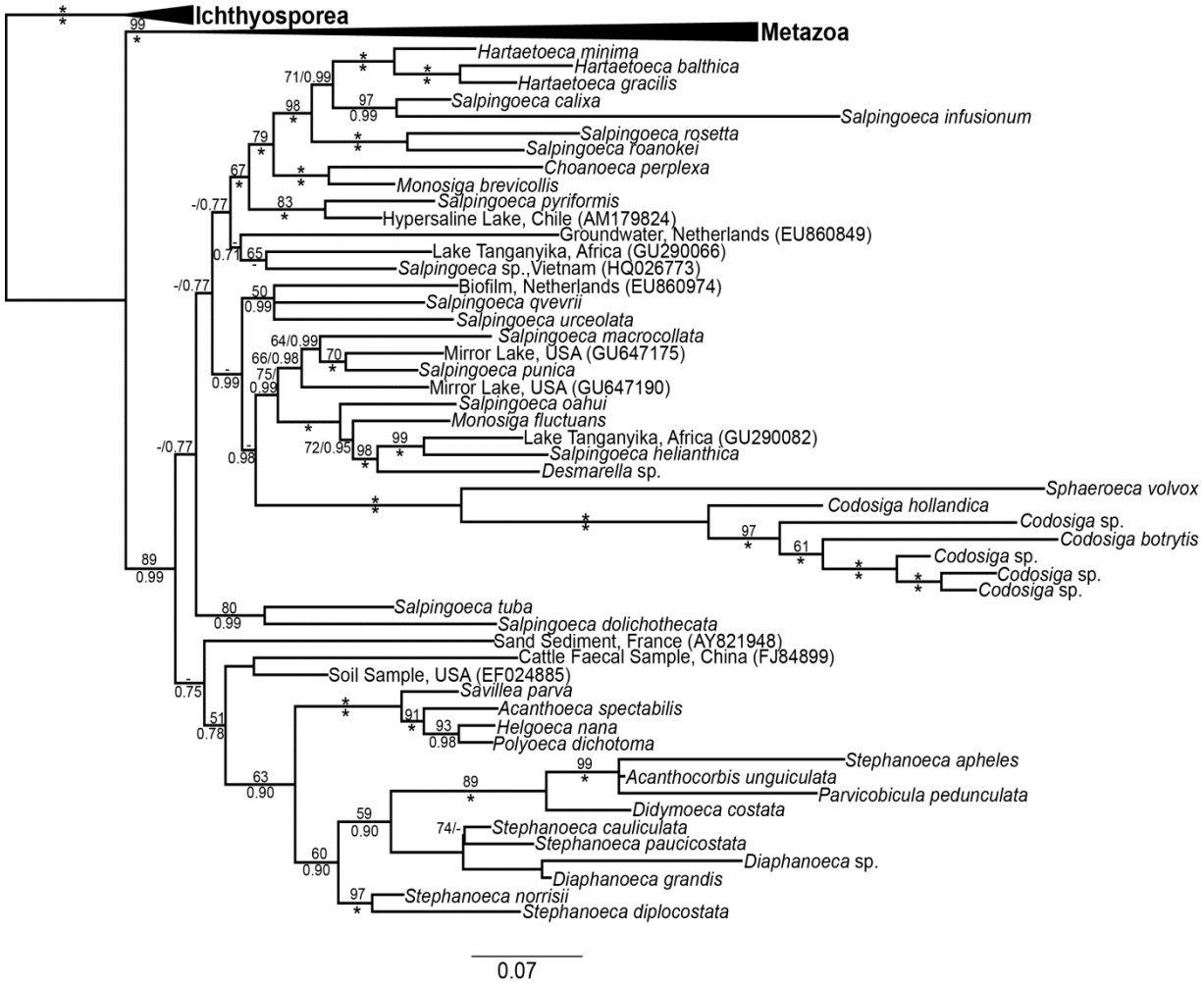
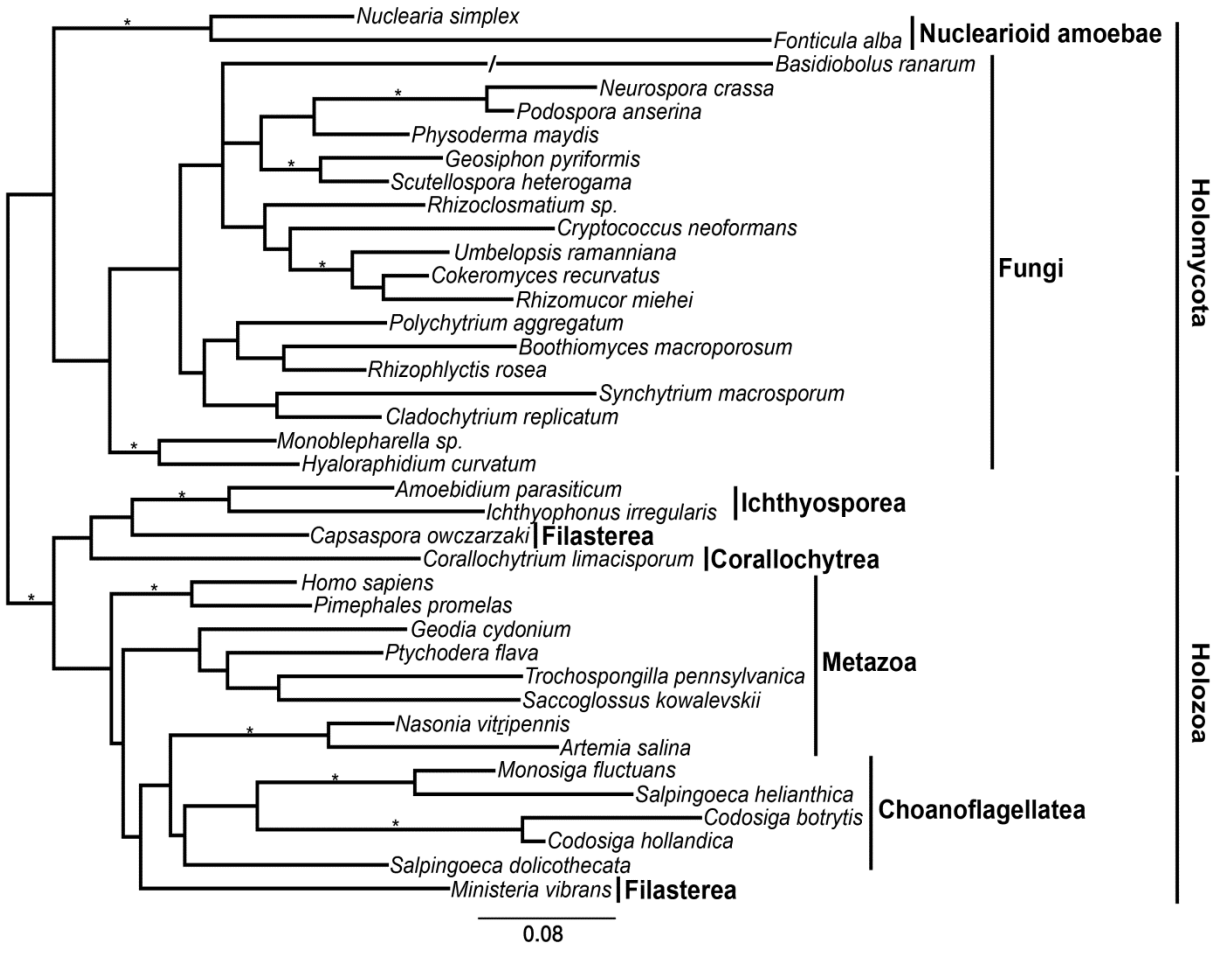
Supplementary Material



Supplement Figure 1. Maximum likelihood phylogeny of the Choanoflagellatea including environmental SSU sequences. Single sequences from each of the 11 putative freshwater clades of del Campo and Ruiz-Trillo (2013) have been added to the alignment used in Fig. 2.



Supplement Figure 2. Maximum likelihood phylogeny of the EF-1A in Opisthokonta. The phylogeny is based upon the conceptual translation of 391 aligned amino acid positions. Branches are drawn proportional to the number of amino acid substitutions per site as indicated by the scale bar at the lower left. Support values of 75% mlBP and 0.97 biPP and above are denoted by an *****.

Supplement Figure 3. Maximum likelihood phylogeny of the EFL. The phylogeny is based upon the conceptual translation of 412 aligned amino acid positions. Branches are drawn proportional to the number of amino acid substitutions per site as indicated by the scale bar at the lower left. The formatting of the tree and labels are the same as Supplement Fig. 2.

Supplement Table 1. Nucleotide sequences used to create HMM for eEF-1A and EFL.

Species	Accession Number
eEF-1A	
Choanoflagellatea	
Codosiga botrytis	HQ896019
Monosiga fluctuans	AY582824
Salpingoeca helianthica	DQ059027
Metazoa	
Homo sapiens	AK222551
Takifugu rubripes	XM_003968818
Filasterea	
Ministeria vibrans	AY582825
Ichthyosporea	
Amoebidium parasiticum	AY582828
Corallochytria	
Corallochytrium limacisporum	AY582826
EFL	
Choanoflagellatea	
Monosiga brevicollis	AY026073
Ichthyosporea	
Creolimax fragrantissima	EU169930
Sphaeroforma arctica	DQ403164
Alveolata	
Heterocapsa triquetra	AY729485
Karlodinium micrum	DQ666284
Perkinsus marinus	XM_002781860
Viridiplantae	
Bathycoccus prasinos	FO082275
Ostreococcus tauri	XM_003082514

Supplement Table 2. Freshwater environmental sequences taken from del Campo and Ruiz-Trillo (2013) included in Supporting Fig. 1.

Clade	Accession Number	Sampling Location
Lagenoeca	HQ026773	Mekong River, Vietnam
Freshwater Choanoflagellates 1	GU290066	Lake Tanganyika
Salpingoeca	GU290082	Lake Tanganyika
Napiformis	GU647175	Mirror Lake, USA
Freshwater Choanoflagellates 2	GU647190	Mirror Lake, USA
Pyxidium	EU860974	Biofilm, Netherlands
Freshwater Choanoflagellates 3	FJ848499	Fecal sample, China
Freshwater Choanoflagellates 4	AY821948	Fresh water clay-sand sediment, France
Clade L	EF024885	Soil, USA
Freshwater Choanoflagellates 5	EU860849	Groundwater, Netherlands
Freshwater Choanoflagellates 6	AM179824	Hypersaline lake, Chile

Supplement Table 3. Sequences used to create EF-1A and EFL phylogenies.

Species	Accession Number
EF-1A	
Choanoflagellatea	
Codosiga botrytis	HQ896019
Codosiga hollandica	**Transcriptome**
Monosiga ovata	AAU94651
Salpingoeca dolichotheca	**Transcriptome**
Salpingoeca helianthica (deposited as *S. amphoridium*)	AAY99757
Metazoa	
Artemia salina	P02993
Geodia cydonium	CAA70221
Geosiphon pyriformis	CAJ75798
Homo sapiens	BAD96271
Nasonia vitripennis	XP_0016056
Pimephales promelas	AAT91089
Ptychodera flava	AAT06192
Saccoglossus kowalevskii	AAT06190
Trochospongilla pennsylvanica	AAZ30697
Filasterea	
Capsaspora owczarzaki	ABD67497
Ministeria vibrans	AAU94652
Ichthyosporea	
Amoebidium parasiticum	AAU94655
Ichthyophonus irregularis	AAL87078
Corallochytrea	
Corallochytrium limacisporum	AAU94653
Nuclearioid Amoebae	
Fonticula alba	ACY78662
Nuclearia simplex	AAU94654
Fungi	
Basidiobolus ranarum	ABB90954
Boothiomyces macroporosum	ABB90947
Cokeromyces recurvatus	AAG28992
Cladochytrium replicatum	ABB90951
Cryptococcus neoformans	XP_568462
Hyaloraphidium curvatum	ABB90950
Umbelopsis ramanniana	AAG29008
Monoblepharella sp.	ABB90949
Neurospora crassa	XP_964868
Physoderma maydis	ABB90944
Podospora anserina	Q01520
Polychytrium aggregatum	ABB90948
Rhizoclosmatium sp.	ABB90945

Rhizomucor miehei	AAG29032
Rhizophlyctis rosea	ABB90961
Scutellospora heterogama	ABB90956
Synchytrium macrosporum	ABB90966
EFL	
Choanoflagellatea	
Acanthoeca spectabilis	**Transcriptome**
Choanoeca perplexa	**Transcriptome**
Diaphanoeca grandis	**Transcriptome**
Didymoeca costata	**Transcriptome**
Hartaetoeca balthica	**Transcriptome**
Hartaetoeca gracilis	**Transcriptome**
Helgoeca nana	**Transcriptome**
Monosiga brevicollis	AAK27413
Salpingoeca infusionum	**Transcriptome**
Salpingoeca macrocollata	**Transcriptome**
Salpingoeca punica	**Transcriptome**
Salpingoeca qvevrii	**Transcriptome**
Salpingoeca roanokei	**Transcriptome**
Salpingoeca rosetta	EGD81537
Salpingoeca urceolata	**Transcriptome**
Savillea parva	**Transcriptome**
Stephanoeca diplocostata	**Transcriptome**
Ichthyosporea	
Creolimax fragrantissima	ABX71668
Sphaeroforma arctica	ABD67498
Sphaeroforma tapetis	ADE62456
Fungi	
Allomyces macrogynus	EC637201
Allomyces arbuscula	ABB84529
Basidiobolus haptosporus	AET35001
Basidiobolus magnus	AET34991
Basidiobolus ranarum	ABB84535
Blastocladiella emersonii	ABK34893
Catenophlyctis sp.	ABB84540
Coelomomyces stegomyiae	ABB84530
Conidiobolus coronatus	BQ622716
Entomophthora muscae	ABB84538
Gaertneriomyces semiglobifer	ABB84533
Geranomyces variabilis	AFN02796
Microallomyces sp.	ABB84542
Olpidium brassicae	ABB84544
Pandora neoaphidis	ADK92169
Powellomyces hirtus	AFN02793
Powellomyces sp.	ABB84539

Powellomycetaceae sp.	AFN02797
Rozella allomycis	ABB84537
Rozella sp.	ABB84536
Spizellomyces punctatus	ABB84531
Spizellomycete sp.	ABB84541
Triparticalcar arcticum	ABB84543
Eukaryota *incertae sedis*	
Leucocryptos marina	BAJ10903
Palpitomonas bilix	BAL63502
Alveolata	
Alexandrium ostenfeldii	HO663348
Amphidinium carteriae	ACF28672
Heterocapsa rotundata	ABV72556
Heterocapsa triquetra	AAV34145
Karenia brevis	EX961328
Karlodinium micrum	ABG56231
Oxyrrhis marina	ABF82158
Perkinsus marinus	XP_002788268
Cryptophyta	
Cryptomonas ovata	BAG54795
Goniomonas amphinema	BAG54796
Guillardia theta	ABF82157
Rhodomonas salina	ABF82160
Euglenozoa	
Diplonema papillatum	ACO50119
Neobodo saliens	ACO50138
Petalomonas cantuscygni	ACO50134
Rhynchopus euleeides	ACO50120
Trypanoplasma borreli	ACO50139
Haptophyceae	
Chrysochromulina sp.	BAG55224
Emiliania huxleyi	CV068986
Isochrysis galbana	AAV34146
Pavlova lutheri	AAV34147
Prymnesium parvum	DV097486
Heliozoa	
Microheliella maris	AFA56271
Raphidiophrys contractilis	BAG54797
Heterophrys marina	AFA56270
Rhizaria	
Bigelowiella natans	ACF24592
Cercozoa sp.	BAJ14653
Chlorarachnion reptans	ACF24594
Chlorarachniophyceae sp.	ACF24593
Collozoum amoeboides	BAM28641

Dictyocoryne profunda	BAM28639
Eucyrtidium acuminatum	BAM28638
Gymnochlora stellata	ACF24595
Lotharella amoeboformis	ACF24596
Lotharella globosa	ACF24597
Lotharella vacuolata	ACF24598
Planoglabratella opercularis	BAG54798
Reticulomyxa filosa	ACF24599
Thaumatomastix sp.	BAJ14652
Rhodophyta	
Callophyllis japonica	BAJ10904
Gracilaria changii	DV963090
Gracilaria sp.	BAJ10905
Grateloupia subpectinata	BAJ10906
Stramenopiles	
Achnanthes kuwaitensis	BAG30802
Asterionella glacialis	BAG30803
Chaetoceros affinis	AFM78111
Chaetoceros didymus	BAG30804
Cylindrotheca closterium	BAG30805
Detonula confervacea	BAG30806
Ditylum brightwellii	BAG30807
Skeletonema costatum	AFM78112
Thalassionema nitzschioides	BAG30809
Thalassiosira pseudonana	XP_002292812
Thalassiosira weissflogii	AFM78113
Viridiplantae	
Acrochaete repens	ACN59926
Acutodesmus obliquus	ACN59931
Bathycoccus prasinos	CCO16363
Bolbocoleon piliferum	ACN59927
Citrus clementina	DY275680
Chlamydomonas incerta	ABA01120
Chlamydomonas reinhardtii	XP_001696568
Chlorella variabilis	EFN56659
Chlorococcum sp.	ABQ81937
Gonium pectorale	BAF99250
Festuca arundinacea	GT044550
Helicosporidium sp. *ex Simulium jonesi*	AAV34148
Lactuca saligna	DW068199
Lactuca virosa	DW157774
Mesostigma viride	ABD58901
Micromonas pusilla	ABQ81939
Nephroselmis olivacea	ACN59934

Ochlochaete hystrix	ACL97364
Ostreococcus lucimarinus	XP_001420985
Ostreococcus tauri	XP_003082562
Parachlorella kessleri	ACN59932
Pleodorina sp.	BAC67663
Polytomella parva	EC750857; EC750063; EC750759
Pyramimonas gelidicola	FS594236
Raphanus sativus	FY444344
Sphaerozoum punctatum	BAM28640
Tetraselmis striata	ACN59933
Tetraselmis tetrathele	ABQ81944
Ulva intestinalis	ABQ81938
Ulva fenestrata	ABQ81945
Urospora sp.	ACL97365
Vigna unguiculata	FF391187
Volvox carteri	XP_002945791

Chapter 5

A comparison of methods to quantify heterotrophic flagellates

Abstract

Heterotrophic flagellates contribute significantly to the matter flux in aquatic and terrestrial ecosystems. Still today their quantification in the various environments bears several problems. Here we face these problems testing and describing different quantification methods, i.e. cultivation methods like the Liquid Aliquot Method (LAM), the live-counting technique, different fixation methods, and a molecular survey called aliquot PCR (aPCR). Each of the described method has its advantages and disadvantages which have to be considered in every single case. With the live-counting technique a detection of living cells up to morphospecies level is possible. Fixation and staining methods are advantageous due to the possible long-term storage and observation of samples. Cultivation methods (LAM) offer the possibility of later molecular surveys and aPCR tools might complete the deficiency of LAM in terms of the missing detection of cultivable flagellates. All these methods have been tested using field samples and cultivated freshwater, marine and freshwater sediment heterotrophic flagellates. In summary, we propose a combination of several techniques closing the gap between the different methodological problems.

Introduction

Heterotrophic flagellates (HF) are a very diverse and heterogeneous group of protists with a size range between 1 and 450 µm. They play an essential role in aquatic and terrestrial food webs as major consumers of bacterial biomass (Arndt et al. 2000; Azam et al. 1983; Bonkowski 2004). However, quantitative data of HF were mostly restricted to estimations of “HNF” (Heterotrophic nanoflagellates = size range of ≤15 µm, Arndt et al. 2000) ignoring the high taxonomic and spatial flagellate variability in aquatic habitats. Thus, quantitative estimations of these protists require accurate estimations of abundance and biomass and a reliable taxonomic resolution. The last issue is especially challenging as the taxonomic affiliation is more difficult for most HF groups (Arndt et al. 2000) in contrast to ciliates (e.g. Foissner and Berger 1996).

Most methods – in the last three decades - were established concerning the use of fixed and stained samples with epifluorescence microscopy (e.g. Caron 1983; Gifford and Caron 2000). However, some attention has to be given to possible negative effects of the used fixatives regarding biovolume changes: Chaput and Carrias (2002) have shown that Lugol’s iodine and glutaraldehyde may cause cell shrinkage in many cases. They concluded the following order of increasing effect on protist cells: formaldehyde < glutaraldehyde < Lugol’s iodine < mercuric chloride. Additionally, a cell loss of up to 56% was reported by Sonntag et al. (2000) for chemically (formaldehyde fixed and DAPI stained and Bouin’s solution fixed and QPS stained) treated flagellates and several studies concentrated on species-specific treatment effects (e.g. Børsheim and Bratbak 1987; Choi and Stoecker 1989; Menden-Deuer et al. 2001).

In contrast, live-counting can be considered as an alternative method to analyse small sample droplets (e.g. Gasol 1993; Massana and Güde 1991). Although this method is difficult regarding a limited available time frame for observation and the need of a certain amount of expertise, it is useful for obtaining high taxonomic morphotype resolutions.

Another possible technique is represented by cultivation methods like liquid aliquot (LAM, Butler and Rogerson 1995) and most probable number method (MPN, Sinclair and Ghiorse 1987). The MPN method was tested for various aquatic but also for soil habitats (Baldock 1986; Ekelund et al. 2011). However, these cultivation techniques are hampered by the fact of the missing detection of uncultivable organisms (Pedrós-Alió 2006). As additionally emphasized by del Campo et al. (2013), the HF groups which have been successfully cultured do not necessarily represent the dominant members in the environment due to the bacterial enrichments. Although being rarely measured in the environment, species like *Cafeteria* spp., *Paraphysomonas* spp. or *Neobodo* spp. may be cultivated in high abundances (e.g. Lim et al. 1999). Thus, culturing bias is an often occurring problem in HF cultivation studies. However, especially in ecological studies the quantification of the most abundant species is highly demanded due to the above mentioned ecological importance of HF. The use of unamended dark incubations to stimulate the naturally occurring bacteria could circumvent this culturing bias problem (Weber et al. 2012).
In the recent years, molecular techniques regarding single protistan cells have increasingly been applied (Jost 2010; Lynn and Pinheiro 2009). A special molecular tool of PCR without a prior DNA extraction represents the aliquot PCR (aPCR). This method was successfully studied in bacteriology (Fode-Vaughan et al. 2001) and clinical research (e.g. Panaccio et al. 1993). To our knowledge aPCR was not practised for ecological surveys regarding protists.

In the present study, we tried to face the problem how to quantify HF in environmental samples. Thus, we compared different traditionally used quantification methods and evaluated the newly established aPCR. The underlying advantages and disadvantages of every method (counting, fixation, cultivation and molecular methods) were elucidated. Hence, we try to give a methodological survey of HF quantification techniques and to provide recommendations of reliable methods regarding different problems. Most of our environmental studies were concentrated on the River Rhine (Germany), as an important HF freshwater habitat to demonstrate the different methods.

Material and Methods

Several different methods to quantify HF have been tested which are listed below. We describe general tools. For reasons of clarity, we separately list special methods which have been used in this study to test specific methodological aspects of quantification. The species and cultivation media used for the different methods are shown in Supplement Table 1.

Counting of live and fixed samples

Counting test

The method of the direct live-counting is a well-proven technique to count flagellates directly under a phase contrast or DIC microscope (e.g. Arndt and Mathes 1991; Gasol 1993; Massana and Güde 1991). For this technique the following steps were used: A prepared microscopic slide was taken to avoid crushing of the HF (see chapter 1) or an Utermöhl chamber (Utermöhl 1958; HydroBios GmbH, Kiel, Germany) with droplets of 5-20 µl of sampling material. For pelagic flagellates undiluted samples and for benthic flagellates a dilution factor of 5 - >20 with filtered river water were generally used (Arndt et al. 2000). A phase contrast or DIC microscope with a magnification of ≥ 200x and the help of high resolution video-recording was utilised for identification. Counting of huge organism amounts was performed in a miniaturized version of a Sedgewick-Rafter cell chamber due to the helpful grid lines. The flagellate composition was analysed within one hour after sampling. For the different live-counting described in this study, we counted 5 µl droplets for each subsample. A long-term routinely survey of pelagic HF in the River Rhine has been performed since the year 2000. We took water samples at the Ecological Rhine Station (Cologne, Germany) every month and live-counted 5x5 µl for four replicates.

For a field sample study with a comparison between different counting investigators, samples were taken at the Baltic Island Hiddensee at the coast of Mecklenburg-Vorpommern (Germany). We took a freshwater sample from a small eutrophic pond in the village Kloster (Reedsaal). A brackish water sample was taken from the Baltic Sea (salinity of 9 PSU). Yeast extract was used for both field samples for two days to reach cell densities high enough for appropriate live-counting. Live-counting was performed as described for the abundance estimation (see above).

Fixation test

For different fixation experiments, we used Schmaltz-Pratt and Pratt medium for marine and freshwater flagellates, respectively. For a study with cultured organisms, subsamples of each culture were preserved with the following commonly used 2% glutaraldehyde (e.g. Caron 1983; Choi and Stoecker 1989; Haas 1982), 2% formaldehyde (e.g. Porter and Feig 1980; Sherr et al. 1989), buffered formaldehyde (e.g. Børsheim and Bratbak 1987; Sherr and Sherr 1983) and 0.5% acidic Lugol´s solution (10g I2, 20g KI, 10g sodium acetate in 140ml aq. dest.) plus 3% formaldehyde (e.g. Sherr et al. 1989). To assure the flagellates were at the same growth stages (an important factor for the size range of the cells (Choi and Stoecker 1989)), we took all organisms for one experiment from the same culture per strain. Five 2 ml replicate subsamples were taken for each of the five different treatments in 10 ml glass test tubes. Another 2ml of ice-cold fixative, glutaraldehyde, formaldehyde and buffered formaldehyde (2% final concentration) freshly prepared in the respective culture media were added to these subsamples. The samples for Lugol preparations were firstly treated with 10 µl concentrated Lugol (0.5% final concentration). After ten minutes the samples were fixed with 2 ml ice-cold formaldehyde (3%) (Sherr et al. 1989). Another five subsamples were used for immediate live-counting.

For the abundance estimation of each culture, droplets of 5 µl of each subsample were live-counted under a microscope. It was sometimes necessary to dilute the live samples with culture medium to a number of about ten flagellates to obtain a better

identification result. Fixed samples were examined within one to six hours after fixation. All samples were examined at 100-400x magnification.

Additionally, a literature survey was carried out trying to complete tested fixation methods regarding different morphotypes/species of flagellates (see Supplement Table 2). For methods applied in these studies see the respective literature: Børsheim and Bratbak (1987), Chaput and Carrias (2002), Choi and Stoecker (1989) and Menden-Deuer et al. (2001). We do not claim having identified all studies regarding fixation methods.

The cell volume dimensions were measured assuming a form of rotation ellipsoid with the cell sizes of length and width. The biovolume was calculated by the cell volume referred to 1 ml.

Recovery test using different methods (live-counting and fixed counting)

For a field study comparison along the River Rhine (Germany), sixteen different sampling points were examined. Three replicate samples were taken with a Ruttner Water Sampler from one metre depth. For live-counting, 30-60 µl of each sample were used to immediately measure abundance and biovolume. For the fluorochrome-counting, 30-80 µl of the samples were fixed with 2% formaldehyde and kept at 4°C in the dark. Within 24 hours, the samples were stained with DAPI (0.1 mg/ml, Porter and Feig 1980) and filtered onto a 0.2 µm membrane filter (see above). The samples were kept at -20°C until abundance and biovolume estimation (see fixation experiments).

Quantification via cultivation approach (LAM) and aPCR

Different media were used to cultivate the HF: For freshwater species Pratt and Wright's Chu medium (Guillard and Lorenzen 1972). The Pratt medium was consisting of 0.1 g/l KNO_3, 0.01 g/l $MgSO_4 \times 7\ H_2O$, 0.01 g/l $K_2HPO_4 \times 3H_2O$ and

0.001 g/l $FeCl_3 \times 6H_2O$ in deionized water. However, WC medium (Guillard and Lorenzen 1972) with its enhanced nutrient supply was most suitable.

For marine HF, Schmaltz-Pratt was prepared of 28.15 g/l NaCl, 0.67 g/l KCl, 5.51 g/l $MgCl_2 \times 6\ H_2O$, 6.92 g/l $MgSO_4 \times 7\ H_2O$, 1.45 g/l $CaCl_2 \times H_2O$, 0.1 g/l KNO_3 and 0.01 g/l $K_2HPO_4 \times 3H_2O$ in deionized water (salinity 35 PSU). The cultures grew in culture flasks (Sarstedt, Nümbrecht, Germany) and an autoclaved wheat grain was added to enhance autochthonous bacterial growth as nutrient source for the HF. Culture flasks were kept under a constant 12/12 h day/night cycle at 10°C.

Liquid Aliquot Method (LAM)

The Liquid Aliquot Method (LAM) (Butler and Rogerson 1995) can be used estimating the quantity of cultivable protists and establishing clonal cultures. It is assumed that all individuals present in a well originate from a single protozoan cell. 1.5 ml WC medium were filled in each well of 24-well plates (Sarstedt, Nümbrecht). One sterilized quinoa grain was provided for each well stimulating the growth of autochthonous bacteria. After a suitable dilution (following volumes can be tested: 1, 2, 6, 10, 12, 15, 20µl), subsamples were added to each well. The presence of HF in the 24-well plates was checked every week during several weeks with the help of an inverted phase contrast microscope (Zeiss Axiovert s100 microscope). At high concentrations of flagellates, abundance estimates were adjusted for possible inoculations of more than one organism in each well assuming a Poisson distribution (Garstecki and Arndt 2000). When flagellates were detected in the 24-well plates during the screening, a volume of 100 µl of the respective well was transferred into tissue culture flasks (Sarstedt, Nümbrecht) filled with 30 ml WC medium and one autoclaved wheat grain for further cultivation and species determination. Flask-cultures provided a higher optical resolution and allowed a long-term storage.

Recovery test using LAM and live-counting in comparison

Additionally, a comparative study of live-counting and LAM of benthic HF in the River Rhine was carried out with sediment samples, which were collected at a sandy sediment station of the River Rhine at Cologne. This routine sampling site is situated on the right riverbank of the River Rhine opposite to the Ecological Rhine-Station of the University of Cologne (Rhine-km 685, 200 m south of the Süd-Brücke). From January 2009 to February 2010 (09/01/2009, 29/01/2009, 03/03/2009, 31/03/2009, 16/04/2009, 07/05/2009, 28/05/2009, 18/06/2009, 16/07/2009, 06/08/2009, 27/08/2009, 17/09/2009, 15/10/2009, 05/11/2009, 03/12/2009, 23/12/2009, 13/01/2010, and 04/02/2010) sediment samples were collected every 3 weeks. For each sample, a volume of 800 µl was taken by pushing a sterilized syringe two times into the sediment up to a depth of 3 mm. The syringe content was transferred to a centrifuge tube and diluted with 10 ml of filtered (<0.2 µm) ambient river water. Sampling was carried out at three sampling sites close to each other (distance about 3-4 m). Live-counting and the LAM method were performed as described above.

Aliquot PCR (aPCR)

According to the liquid aliquot method, 96-well PCR plates (Sarstedt, Nümbrecht, Germany) were used for molecular survey. An appropriate dilution of the sample water was added to the plates ensuring that only one eukaryotic cell was present in each well for amplification. For the amplification, a final template volume of 80 µl was used which was deep-frozen at -80 °C prior to PCR reactions. For the appropriate PCR preparations and procedures see below.

Recovery test using aPCR, LAM and live-counting in comparison

We tested three different cultured heterotrophic flagellates regarding their recovery using aPCR (*Apsuomonas proboscidea*, *Spumella* sp., *Thaumatomonas coloniensis*). For the preparation of cell suspensions, small aliquots (3-10 µl) of HF cultures were

counted under an inverted microscope (Zeiss Axiovert s100, n=10). We diluted the flagellate cultures to a reduced number of individuals per volume under the laminar flow. For each of three replicates a total volume of 16 ml was separated in 80 µl aliquots and filled in sterile reaction tubes (0.5 ml). Afterwards, the samples were deep-frozen at -20°C. The PCR reaction performed in the 80 µl aliquots was done according to the above described procedure. However, instead of 1.25 and 1,5 mM $MgCl_2$, 2.5 mM $MgCl_2$ was used for these amplification and reamplification steps. We used the D5-Rev-n primer (Wylezich et al. 2010) for sequencing reactions. Regarding aPCR recovery tests of *Thaumatomonas coloniensis*, reamplification was also tested with higher template volumes than 1 µl (20 tests with 5 µl and 14 µl of template volume respectively).

For a comparative study of live-counting, LAM and aPCR, pelagic HF samples were taken from the River Rhine at the Ecological Rhine Station of the University of Cologne (sampling depth: 0.5 m) on three different sampling dates: 08/05/2008, 19/06/2008, and 10/07/2008.

For the live-counting technique, HF abundances of three replicates were analysed for each sampling. 5 µl aliquots were taken after carefully shaking of the sampling bottles to reduce settlement effects. Five aliquots of each replicate were counted according to the above described live-counting method. The LAM method was performed as explained above. For the aPCR, 96-well plates were prepared (see above). WC medium was used for the dilution of Rhine water getting a final template volume of 80 µl for PCR. The samples were deep-frozen at -20°C prior to PCR. For amplification of D1-D5 region of the LSU rDNA, we used the universal eukaryotic primers fw1 (Sonnenberg et al. 2007) and D5-Rev2 (Wylezich et al. 2010). The PCR reaction mix contained 0.2 mM dNTPs, 1.25 mM $MgCl_2$, 1x PCR buffer containing KCl, ddH_2O, 0.1 µM of each primer and 1 unit of Taq-Polymerase (MBI Fermentas, St. Leon-Rot, Germany). PCR was performed with the following steps: denaturation step at 95°C for 2 min, followed by 35 cycles with 30 s at 95°C, 45 s at 50°C and 90 s at 72°C. The PCR product served as template for a following reamplification of the D3-D5 region of LSU rDNA. The reactions were conducted in 25 µl comprising 1µl of the PCR product,

0.1 µM of each primer – D3-For-n and D5-Rev-n (Wylezich et al. 2010) - 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 1x PCR buffer containing KCl and 0.5 units of Taq-Polymerase. Reamplifications steps were the same as done for the amplification. The sequencing was carried out using the Big Dye Terminator Cycle Sequencing Kit (version 3.1, Applied Biosystems, Darmstadt, Germany) and the D3-For-n primer and sequenced on an ABI 3100 automated sequencer.

Statistical analysis

We tested the results regarding significant statistical differences by ANOVA followed by a Dunnett post-hoc test. Significant differences in abundances were estimated using a Tukey post-hoc test. The statistical differences between each counting person (Fig.1) were calculated by ANOVA followed by a Tukey post-hoc test. The results of these statistical estimations regarding the fixation experiments are shown in Table 1.

Results

Counting of live and fixed samples

Counting test

Within our long-term study (period of 2000-2014) of HF in the River Rhine the following main groups of HF have been observed and are thus generally detectable: Apusomonads, bicosoecids, cercomonads, choanoflagellates, chrysomonads, ciliophryids, cryomonads, cryptophyceans, dinoflagellates, euglenids, glissomonads, jacobids, kathablepharids, kinetoplastids, *Multicilia* sp., protista incertae sedis (*Paramastix* sp., *Quadricilia* sp.), spironemids, thaumatomonads (systematics according to Adl et al. 2012, data by H. Arndt, unpublished long-term data). With the live-counting method, it is thus possible to detect the different features of recognition in the different flagellate groups: The number of flagella (e.g. important for short

second flagellum like *Spumella* sp. has), specific modes of movement (especially important for e.g. free-living kathablepharids (bodonids), some euglenids), the presence of specific cell particles like e.g. ejectisomes (e.g. present in cryptophyeans like *Goniomonas* sp.).

Regarding the field sample study with a comparison of different counting investigators, no significant differences have been observed. Hence the abundance of counted HF varied only slightly between the different counting investigators, irrespective of brackish or freshwater samples (see Fig. 1).

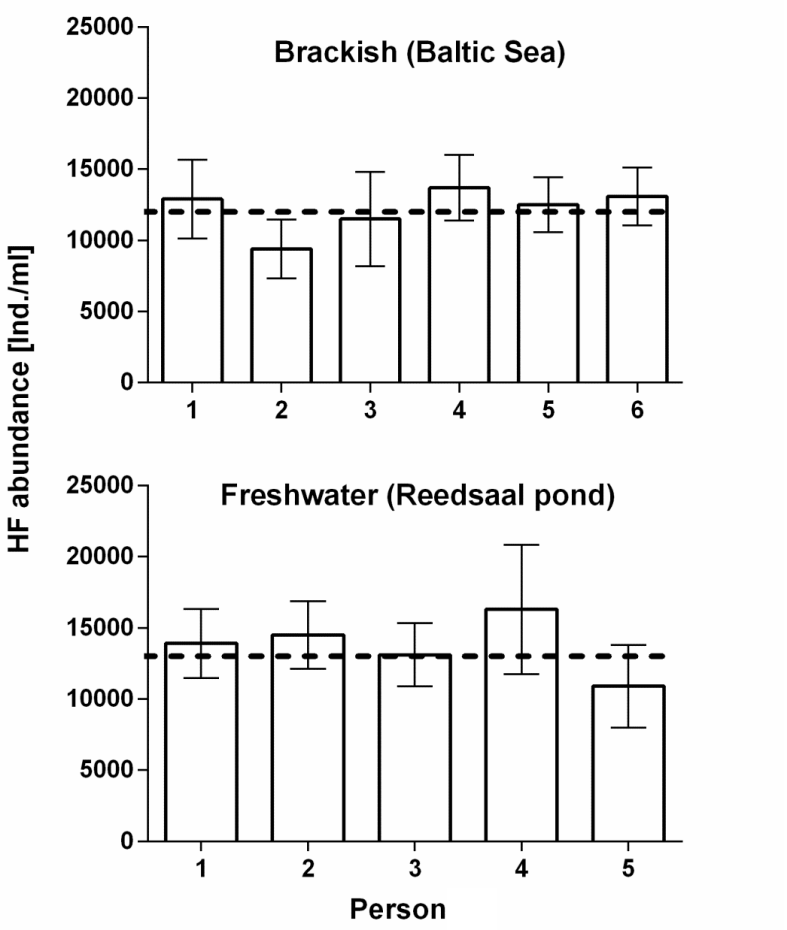


Figure 1. Comparative HF live-counting of different persons (1-6) for brackish and freshwater samples, respectively. Each bar represents counting of one person. The average of all live-counting is shown by the dashed line.

Fixation test

The results of cell volume and abundance of the fixed HF cultures used in this study are listed in Table 1. A summary of literature and own data of different flagellates regarding the percentage of live cell volume is given in Supplement Table 2.

Table 1. Results of average cell volumes and abundance estimations of fixed HF.

	Average % live cell volume (± 95% confidence interval, 20-40 organisms measured)				Average % live flagellate abundance (± 95% confidence interval, 5 replicates)			
Species	**G**	**F**	**bF**	**LF**	**G**	**F**	**bF**	**LF**
Apusomonas proboscidea	62 ± 26 [sh]	61 ± 38 [sh]	78 ± 26	128 ± 52 [sw]	63 ± 26 [l]	61 ± 38 [l]	78 ± 26	128 ± 52
Neobodo designis	62 ± 14 [sh]	73 ± 12	70 ± 14 [sh]	97 ± 17	56 ± 17 [a]	38 ± 19 [a,l]	15 ± 9 [a]	62 ± 23
Bodo saliens	58 ± 16 [sh]	67 ± 8 [sh]	81 ± 14	86 ± 11	135 ± 41	121 ± 23	118 ± 46	121 ± 18
Bodo saltans	26 ± 4 [sh]	22 ± 4 [sh]	26 ± 6 [sh]	95 ± 13	170 ± 45 [a,b]	106 ± 47	98 ± 49	119 ± 12
Bodo sorokini	50 ± 9 [sh]	74 ± 12 [sh]	63 ± 13 [sh]	64 ± 10 [sh]	65 ± 16 [a]	49 ± 18 [a,b]	83 ± 22	68 ± 7 [a]
Cercomonas crassicauda	64 ± 23 [sh]	44 ± 8 [sh]	56 ± 13 [sh]	59 ± 25 [sh]	35 ± 12 [a]	16 ± 11 [a,l]	17 ± 10 [a,l]	54 ± 16 [a]
Cercomonas sp.	30 ± 7 [sh]	33 ± 7 [sh]	35 ± 7 [sh]	27 ± 4 [sh]	62 ± 8 [a]	51 ± 10 [a,l]	57 ± 15 [a]	74 ± 16 [a]
Chilomonas paramecium	68 ± 7 [sh]	0 [sh]	0 [sh]	85 ± 8 [sh]	97 ± 27	0 [a, g, l]	0 [a, g, l]	108 ± 13
Codosiga hollandica	86 ± 16	84 ± 22	244 ± 81 [sw]	159 ± 52	66 ± 23	93 ± 61	69 ± 22	52 ± 32
Crypthecodinium cohnii	68 ± 13	74 ± 14	75 ± 12	85 ± 14	124 ± 15	125 ± 9	125 ± 13	102 ± 32
Entosiphon sulcatum	64 ± 9 [sh]	125 ± 12 [sw]	106 ± 15	117 ± 15	80 ± 15	67 ± 31	109 ± 46	103 ± 55
Ochromonas sp.	38 ± 12 [sh]	29 ± 6 [sh]	67 ± 15 [sh]	72 ± 13 [sh]	89 ± 10	105 ± 19	85 ± 26	76 ± 25 [f]
Oxyrrhis marina	47 ± 9 [sh]	41 ± 7 [sh]	38 ± 7 [sh]	44 ± 8 [sh]	124 ± 19	112 ± 34	52 ± 19 [a, f, g, l]	150 ± 37
Percolomonas cosmopolitus	149 ± 55	80 ± 15	68 ± 10	85 ± 15	115 ± 30	137 ± 47	141 ± 69	151 ± 72
Pseudobodo tremulans	50 ± 11 [sh]	47 ± 8 [sh]	60 ± 8 [sh]	67 ± 11 [sh]	119 ± 78	84 ± 10	63 ± 29	88 ± 17
Salpingoeca euryoecia	99 ± 24	118 ± 31	141 ± 25	73 ± 17 [sh]	51 ± 26	39 ± 9	59 ± 35	26 ± 22 [a]
Salpingoeca limnea	62 ± 9 [sh]	67 ± 13 [sh]	105 ± 24	103 ± 26	65 ± 28 [a]	75 ± 61 [a]	41 ± 11 [a]	64 ± 53 [a]
Salpingoeca sp.	107 ± 27	153 ± 33	115 ± 23	171 ± 57	76 ± 30	97± 88	104 ± 77	97 ± 46
Spumella sp.	71 ± 12 [sh]	65 ± 11 [sh]	94 ± 15	89 ± 14	96 ± 19	105 ± 13	99 ± 27	111 ± 30
Stagondion pyriformis	138 ± 38	146 ± 37	138 ± 32	161 ± 38 [sw]	85 ± 61	99 ± 101	97 ± 92	63 ± 16

Abbreviations: **G**=glutaraldehyde, **F**=formaldehyde, **bF**=buffered formaldehyde, **LF**=Lugol´s fixative + formaldehyde; [sh] significant shrinkage ($p<0.05$), [sw] significant swelling ($p<0.05$), significant difference in abundance ($p<0.05$) in comparison to: [a] live counting, [b] buffered formaldehyde, [g] glutaraldehyde, [f] formaldehyde, [l] Lugol´s fixative + formaldehyde.

Various effects for the different species have been obtained regarding the abundance (Table 1). For the other half no significant changes in abundance were observed and for just one species a significant increase was obtained ($p<0.05$, *Bodo saltans* for glutaraldehyde). Half of all tested species showed a significant loss ($p<0.05$) for at least one of the tested fixatives. The dinoflagellate *Oxyrrhis marina* was the only species showing losses regarding one fixative (buffered formaldehyde). The other dinoflagellate tested (*Crypthecodinium cohnii*) was not significantly affected by any fixative. Cercomonads were very sensitive to all fixatives, with significant losses (16% - 74% of live abundance). We observed a loss for *Cercomonas* sp. (57%) and even higher losses for *Paracercomonas crassicauda* (17%) using buffered formaldehyde. In general, formaldehyde, unbuffered or buffered, revealed the most drastic losses of HF abundance (*Neobodo designis* (38% and 15%, respectively) and *Chilomonas paramecium* (0% and 0%, respectively). The cells of *Chilomonas paramecium* could sometimes still be identified after fixation by buffered and unbuffered formaldehyde, though we observed plasma membrane damage. The kinetoplastids in general were differently affected by fixatives. Even between closely related species, the results differed significantly for *Bodo saliens* and *Bodo saltans*. Significant abundance losses of *Neobodo designis* occured using glutaraldehyde (56%), formaldehyde (38%), and buffered formaldehyde (15%), respectively. *Procryptobia sorokini* abundance decreased for glutaraldehyde (65%), formaldehyde (49%) and, as a distinction from *Neobodo designis*, with Lugol + formaldehyde (68%), respectively, but not for buffered formaldehyde.

We investigated five choanoflagellates from marine and freshwater habitats with different theca forms (organic covering), without theca and/or the ability to form colonies. The colony forming freshwater species (*Salpingoeca limnea*) showed significant differences in abundance for all fixatives (glutaraldehyde (65%), formaldehyde (75%), buffered formaldehyde (41%), Lugol + formaldehyde (64%)) compared to live counting. Besides, the only other choanoflagellate species affected by a fixative (Lugol + formaldehyde (26%)) was the non-thecate species *Salpingoeca euryoecia*.

Additionally, we compared the different used fixatives (literature and own data) concerning different groups of HF (see Fig. 2). However, 72% of all tested organisms showed cell volume shrinkage (22%-99% of live cell volume) irrespective of the used fixative. Among these, 100% of tested bicosoecid, chrysomonad, kinetoplastid, cryptomonad and cercomonad species showed shrinkage of cell volume. Within the dinoflagellates, we observed 17 of 42 totally tested organisms with a cell volume of ≥ 100%, under which 11 (26% of total tested dinoflagellates) were showing cell volume swelling (≥ 105%). Regarding the choanoflagellates, 13 of 20 organisms (65%) indicated a cell volume ≥ 105%. To conclude, no clear patterns either of swelling or shrinkage or of the used fixation methods could be detected regarding the effects on cell volume.

Recovery test using different methods (live-counting and fixed counting)

For the field study comparison along the River Rhine (Germany), the abundances obtained by live-counting reached 250-600 HF/ml, whereas 50-250 HF/ml were calculated for the fluorochrome-counting (Fig. 3). The mean fluorochrome-counting deficiency was 66% for the River Rhine. The counting differences even increased for the biovolume estimations (Fig. 3). Higher biovolumes were detected using live-counting (mean size range of 20-70 μm^3/l), whereas the mean size for fluorochrome-counting ranged around 10 μm^3/l. The corresponding taxonomic composition of HF during the same sampling time is shown in Scherwass et al. (2010).

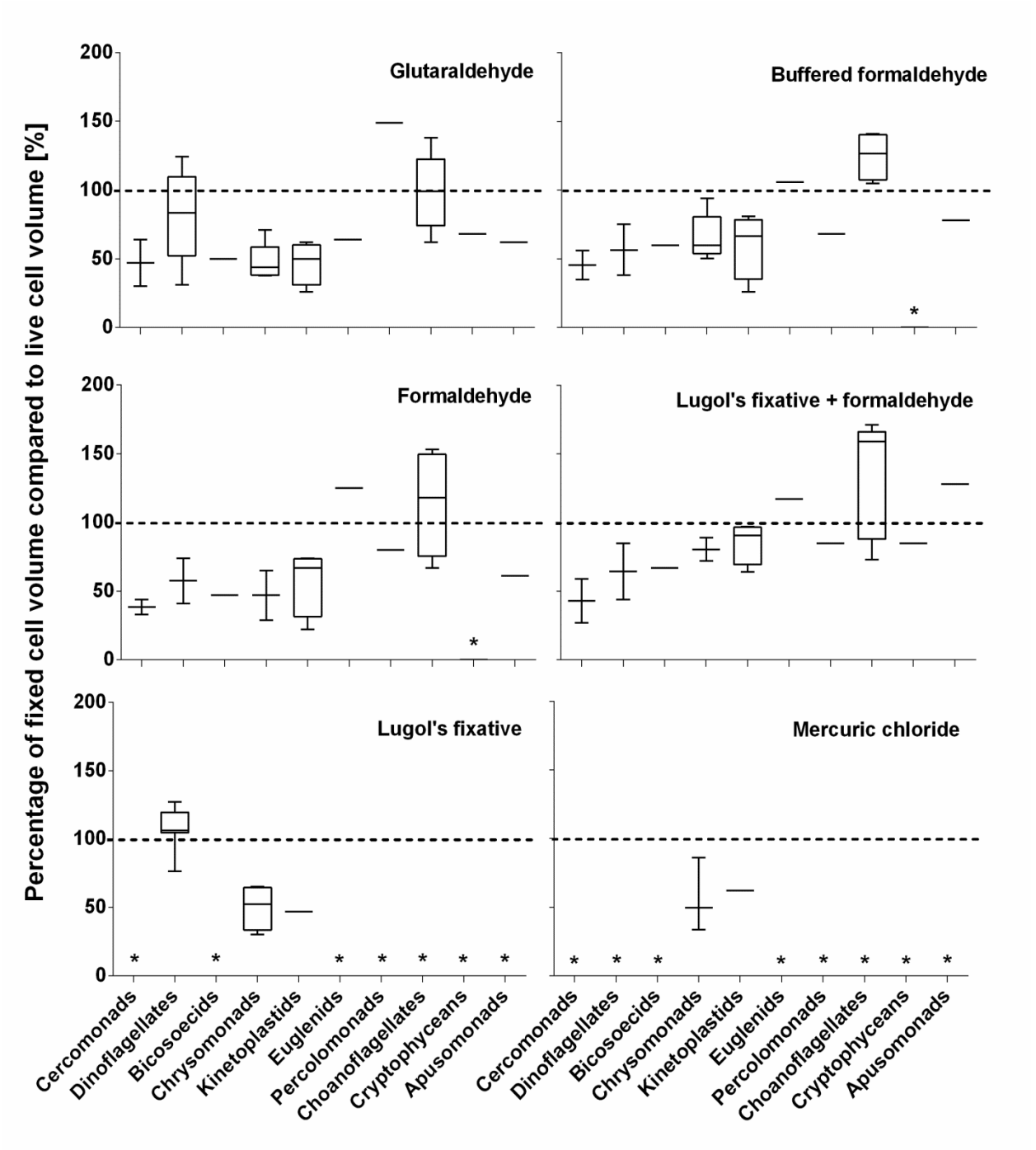


Figure 2. Comparison between different used fixatives for different taxonomic groups of HF. Data obtained from literature (see Supplement Table 2 in Supplementary Material) and own studies (Table 1). Single data points represent data with no replicates. * indicates no available data for the group.

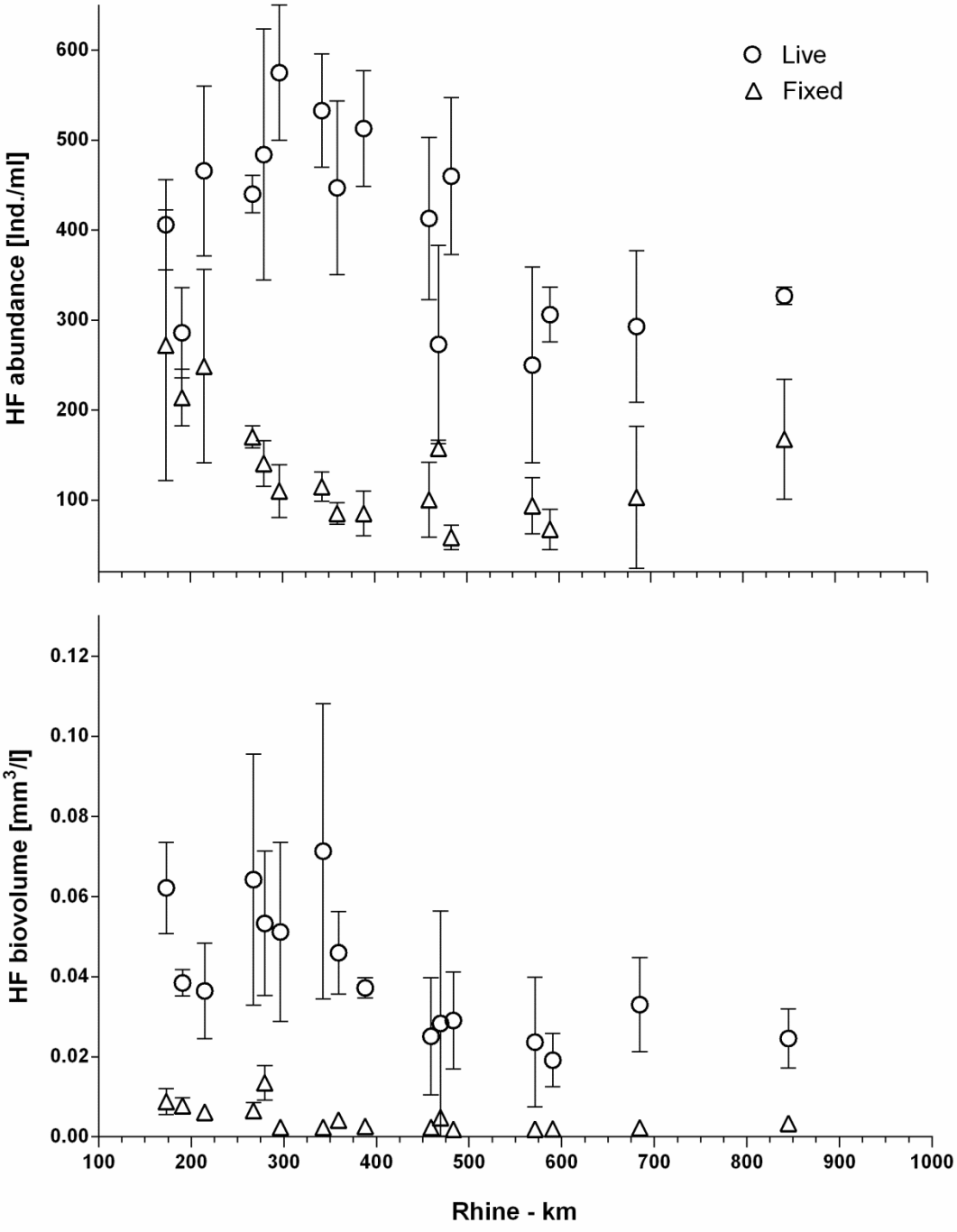


Figure 3. Comparison of HF-abundances and biovolumes obtained by live-counting and fluorochrome-counting (fixed) along the River Rhine.

Quantification via cultivation approach (LAM) and aPCR

Recovery test using LAM and live-counting in comparison

The investigation of samples taken in the River Rhine using the LAM resulted in seven different taxonomic groups of HF (ancyromonads, apusomonads, bicosoecids, cercomonads, choanoflagellates, chrysomonads, kinetoplastids). Using live counting, one additional group (cryptomonads) could be detected.

Regarding benthic HF, a mean of about 10% of the live-counted HF was recovered using LAM (see Fig. 4). The 10% recovery is in the range of the LAM using pelagic HF (see following part).

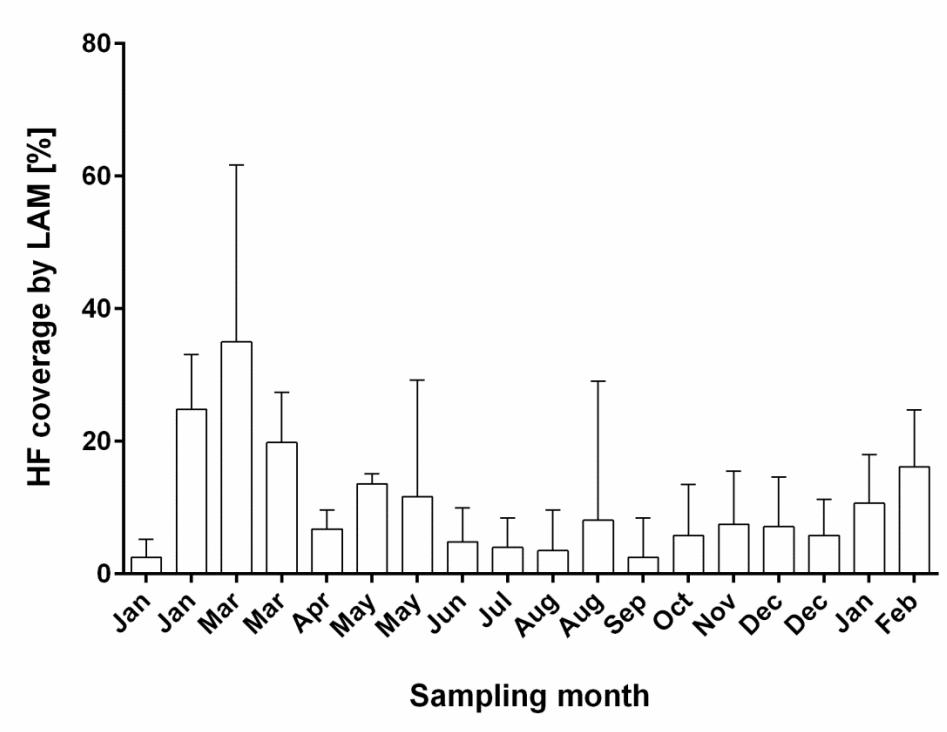


Figure 4. Recovery of benthic HF in the River Rhine cultivated by Liquid Aliquot Method (LAM) as percentage of live-counting.

Recovery test using aPCR, LAM and live-counting in comparison

The aPCR did not show positive results. Therefore we optimized this approach using cultures of HF by carrying out a nested PCR.
The recovery efficiency of the three tested flagellate cultures showed species-specific variability (see Table 2).

Table 2. Recovery test of aliquotPCR using three different flagellate cultures.

Culture	Expected result [Ind./ml]	Obtained result [Ind./ml]	Efficiency [%] ± SD
Apusomonas proboscidea	2.5	1.46	58.3 ± 50.52
Spumella sp.	2.1	0.94	45 ± 43.6
Thaumatomonas coloniensis	2.1	0.31	15 ± 14.88

The recovery for *Apusomonas proboscidea* resulted in 58.3%, whereas 45% of *Spumella* sp. was rediscovered by aPCR. Although higher volumes (5 µl and 14 µl as template for the nested PCR) were also tested regarding *Thaumatomonas coloniensis*, no more positive results could be obtained than tested for 1 µl template volume (15%). All three recovery tests showed high standard deviations.

Concerning the River Rhine samples, we were able to detect five different taxonomic HF groups in total (bicosoecids, cercomonads, choanoflagellates, chrysomonads, cryptomonads, kinetoplastids) using all three methods (live-counting, LAM cultivation method and aPCR). The aPCR approach did not rediscover apusomonads and

ancyromonads. Using the LAM, cryptomonad species were not recovered as already described above. The highest abundances of pelagic HF were achieved by live-counting (see Fig. 5). We achieved 10% of the live counted HF using the LAM and 22% using aPCR (mean of all three months, Fig. 5).

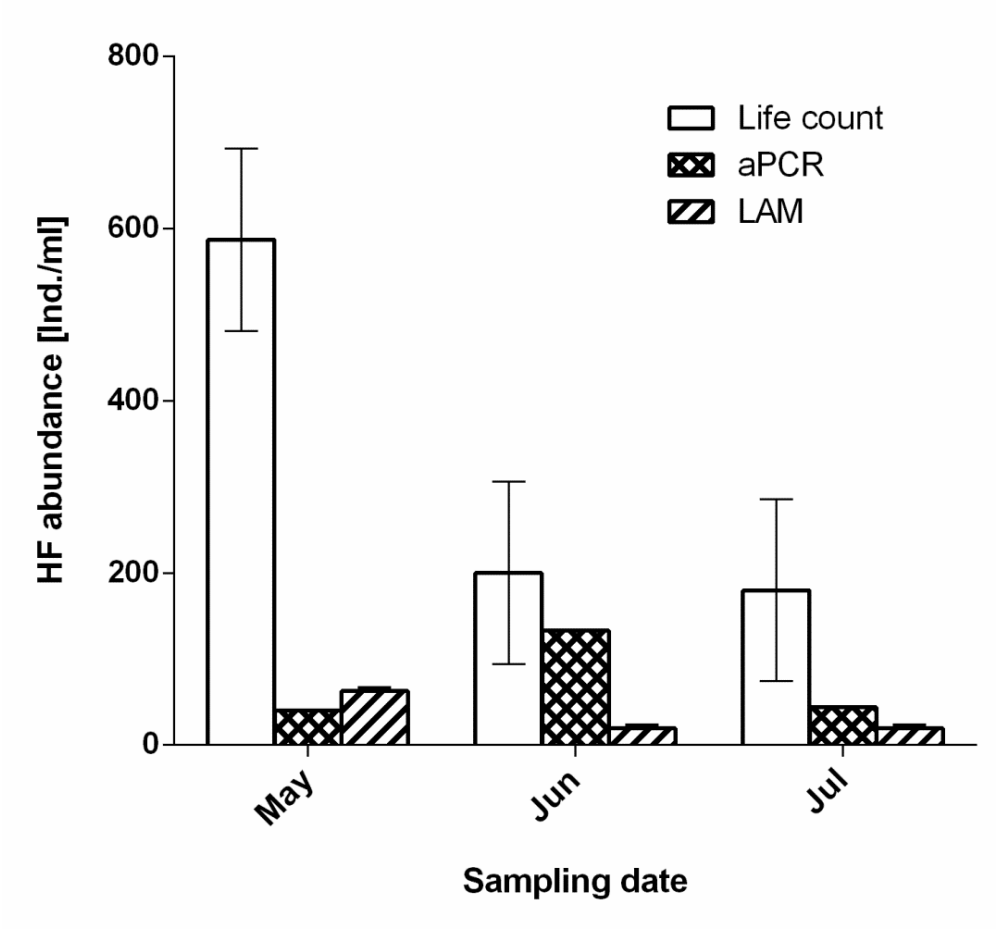


Figure 5. Comparison of HF-abundances obtained by live-counting, aliquot PCR (aPCR) and Liquid Aliquot Method (LAM) regarding three different sampling dates in the River Rhine (mean ± SD, n=3 for live-counting and LAM).

Discussion

In the present study, we investigated and compared different quantification methods to study heterotrophic flagellates. The aim was to find a fast and usable method to obtain reliable results comparing the advantages/disadvantages of different methods (Table 3).

Within the last three decades the use of quantification methods was mostly concentrated on using fixation methods. Yet, this approach is problematic: Cell losses in terms of shrinkage and swelling and abundance loss have been reported for different taxonomic protist groups obtained by different fixation studies (Børsheim and Bratbak 1987; Chaput and Carrias 2002; Menden-Deuer et al. 2001; this study, Fig. 2). The counting of fixed HF revealed lower abundances and biovolumes compared to live-counted HF (Fig. 3). This might be caused by the above mentioned cell losses due to fixation processes. The storage (in a freezer) of fixed and stained samples could additionally cause underestimated cell abundances and biovolumes due to autofluorescence loss (Bloem et al. 1986). However, the general possibility of sample storage and the potential long-time sample observation is advantageous.

Besides, it does not seem that the preferred habitat (marine or freshwater) or cell membrane associated structures are responsible for a possible cell resistance against distortion or disruption by fixatives, e.g. choanoflagellates with a theca (organic cell covering) did not seem to be less affected by the fixatives than those without a theca (compare Table 1). This finding is in agreement with Sonntag et al. (2000) who could not find a cell protection effect by organic cell coverings. In addition, they neglected a single conversion factor for flagellates because the species-specific reactions regarding different fixatives could not be considered. Hence, they suggested interpreting adequate conversion factors as carefully as possible.

Table 3. Advantages and disadvantages of different HF quantification methods.

Method	Advantage	Disadvantage
Live counting	Identification to morphotypes/species level possible	Short time frame for observation/identification, limited amount of samples manageable
Cultivation (Liquid Aliquot Method)	Species obtainable for further studies, e.g. molecular and detailed morphological studies	Coverage of only cultivable species (generally less than 10% of live counts)
Fixation/Staining	Storage of samples, long time frame for observation	Cell losses, species-specific artefacts of fixatives
Aliquot PCR	Sequence data available, cultivation independent, high taxonomic resolution	Specific primers required, sequencing biases, time-consuming and expensive
Flow cytometer	Rapid and reliable counting of cells	Staining required, species detection difficult, long adaptation to specific sample characteristics. expensive equipment
FISH	Accurate and time-saving method, when specific probe is available	Specific probes are still missing for several groups, probe design required
Next Generation sequencing	Huge sequence dataset, cultivation independent	Primers required, active/inactive forms not detectable, different rRNA copy numbers for protists, PCR bias, incomplete databases allow only a low percentage of sequences to be assigned

Apart from fixation methods, flow cytometric measurements are rapid and reliable enumerating techniques. However, flow cytometry is advantageous given that appropriate cell staining techniques are already approved, no high-resolution taxonomic detection is required, and high costs are irrelevant (Christaki et al. 2011; Vives-Rego et al. 2000).

In addition, the applications of next-generation sequencing and DNA-barcoding for HF field studies have increased over the last years (e.g. Egge et al. 2013; Pawlowski et al. 2011; Stoeck et al. 2010). Yet, some problems still remain unresolved like specific instead of wide-range primers, different rRNA copy numbers for protists, PCR biases, the difficulty of differentiating active from inactive forms (e.g. cysts), and incomplete databases with high error numbers (Weber and Pawlowski 2013; Will and Rubinoff 2004; Prosdocimi et al. 2013). As an alternative to cultivation and metagenomic methods, single cell genomics have been developed in the last years being able to detect metabolic features and general structures and dynamics of natural microbial communities (Heywood et al. 2010; Stepanauskas 2012).

FISH (Fluorescence *in situ* hybridisation) is another very important HF quantification technique (e.g. Massana et al. 2002, 2006). Unfortunately, specific probes are still missing for several flagellate groups. Furthermore, the long process of developing FISH probes (getting enough sequence information of target groups, designing and evaluating probes) hamper a fast quantification of flagellates in environmental samples. This is rather difficult for a fast quantification of environmental samples.

As elucidated, it is highly demanded to find a suitable alternative quantification method. The live-counting technique covered all main HF groups in the present study. The groups of diplomonads and preaxostylan species were not found as they are typically occurring in anoxic environments. Our data are in good agreement with data from Weitere and Arndt (2003) who recovered all main HF groups investigating two sampling points of the River Rhine. Besides, the reproducibility of this method was confirmed by our test with different counting investigators (Fig. 1, compare Gasol 1993). Hence, an important advantage of the live-counting method is the high coverage of the main groups and a possible differentiation up to morphospecies level.

In addition, live-counting of samples consisting of high detritus amounts minimises the risk of missing HF masked by the detritus particles as it is the case for other quantification techniques. Furthermore, a possible classification to different functional traits is enhanced.
As a major disadvantage, only a limited time-frame for observation is available. The whole procedure of differentiating all taxonomic HF features and scanning huge amounts of non-concentrated samples is very time-consuming (Sime-Ngando et al. 1990).

The LAM cultivation method is a well-established technique (Butler and Rogerson 1995). Rather problematic is the fact, that cultivation methods cover only the actively bacterivorous HF (compare Massana and Güde 1991 for MPN) and underestimate all non-cultivable protists (Pedrós-Alió 2006). *Paraphysomonas* spp. and *Oikomonas* spp. are for example highly bacteria feeding HF and are thus able to outcompete other species which were originally more abundant in the environment (Del Campo et al. 2013). Cryptomonads have been the only HF group not recovered using LAM. A careful explanation might be that this group belongs to those HF groups not as easy cultivable as others. The application of cultivation methods should thus be considered critically. However, the possible cultivation of the sampled protists for further experiments is advantageous.

The molecular aPCR method - hitherto used for prokaryotes - was newly established for ecological habitat investigations of HF in this study. This method is useful for investigations with varying densities of protists ranging from 1 to 10,000 cells/ml, thus offering the possibility to detect low to high concentrated HF. We diluted samples from enriched habitats securing one individual cell in each aliquot, and thus avoiding the occurrence of chimeras (Brakenhoff et al. 1991; Liesack et al. 1991; Richards and Bass 2005). Working with sample volumes of up to 80 µl for the PCR (100 µl final volume) allowed us investigating very low concentrated samples concerning flagellates (<2-3 cells/ml), hence aPCR is a kind of single-cell PCR without the use of micromanipulation. One major advantage of aPCR is the increased taxonomic

resolution. However, two flagellate groups (apusomonads and ancyromonads) were not detected by aPCR. A possible explanation for this might be that those groups are occurring in lower abundances in the River Rhine (compare Weitere and Arndt 2003). Even though reamplification - as performed for the aPCR - bears a contamination risk (Alvarez-Martinez et al. 2006), the risk of chimeras has to be neglected using single cells - in contrast to the risk using DNA from environmental samples.
Typical disadvantages as discussed for single-cell PCR are also applied for aPCR as the need of group-specific primers and no possibility of recourse of already used cells (Lynn and Pinheiro 2009).

The LAM and aPCR methods were compared with live-counting in the present study for benthic and pelagic HF in the River Rhine (Fig. 4 and Fig. 5). Regarding live-counting, the highest abundances were gained in comparison to the LAM method and aPCR. LAM revealed a mean of 10% recovery of the live counted HF for both, pelagic and benthic HF. This is confirmed by Caron et al. (1989) who proposed a Most Probable Number (MPN) method to direct count method ratio of >10%. In contrast, aPCR reached about 22% of the received live counted pelagic HF abundance (mean of all three months, Fig. 5). This is approximately 10% higher than using LAM, which is due to the coverage of non-cultivable protists by the aPCR in contrast to the LAM.

Concluding Remarks

It was shown, that every tested and described quantification method has specific advantages and disadvantages. Fixation and staining methods offer the possibility of long-term sample storage and observation. The live-counting method is advantageous in terms of recognising living cells with their species-specific behaviour and morphology. The advantage of cultivation methods like LAM is the possible use of the cultivated species for later molecular studies (e.g. Scheckenbach et al. 2005, 2006) and the aPCR technique allows analysing genotypes by obtaining sequence data.

The choice of method is thus, as always, depending on the study aim and should be chosen carefully regarding the underlying question. Several combined methods (at least two methods) are often most suitable for obtaining reliable results (e.g. Auinger et al. 2008; Caron et al. 1989). We recommend using the live-counting technique combined with fixation methods when having only a limited time frame available. The problem of possible shrinkage/swelling should be considered when calculating biovolumes. The functional importance of HF can be best observed using the live-counting technique as different functional traits can be distinguished by this method (e.g. different pelagic or benthic feeding types). For the species diversity of a habitat, we recommend using culture-independent molecular tools such as clone libraries and next-generation sequencing methods (with group-specific primers, depending on the question) combined with LAM for further cultivation of detected species. In addition, all non-cultivable HF missed by the LAM (compare Jost 2010) can be detected by the aPCR. Thus, one method is capable closing the gap of the other.

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Supplementary Material

Supplement Table 1. Species used in the experiments.

Species	Taxonomic group	Medium	Method
Apusomonas proboscidea Alexeieff 1924[1)]	Apusomonads	P	F
Apusomonas proboscidea Alexeieff 1924	Apusomonads	WC	Aliquot PCR
Bodo saliens Larsen and Patterson 1990	Kinetoplastids	SP	F
Bodo saltans Ehrenberg 1832[1)]	Kinetoplastids	P	F
Cercomonas sp. [1)] (Dujardin) Karpov et al. 2006	Cercomonads	P	F
Cryptomonas (Chilomonas) paramecium (Ehrenberg 1831) Hoef-Emden and Melkonian 2003[4)]	Cryptomonads	P	F
"Codosiga hollandica" Carr, Richter and Nitsche [1) 6)]	Choanoflagellates	P	F
Crypthecodinium cohnii (Seligo) Javornicky 1962[2)]	Dinoflagellates	SP	F
Entosiphon sulcatum Stein 1878[3)]	Euglenids	P	F
Neobodo designis (Skuja) Vickerman 2004	Kinetoplastids	SP	F
Ochromonas sp. Wyssotzki 1887	Chrysomonads	P	F

Oxyrrhis marina Dujardin 1841[2)]	Dinoflagellates	SP	F
Paracercomonas (=Cercomonas) crassicauda (Dujardin) Bass and Cavalier-Smith 2009[1)]	Cercomonads	P	F
Percolomonas cosmopolites (Ruinen) Fenchel and Patterson 1986	Percolomonads	SP	F
Procryptobia (=Bodo) sorokini (Zhukov) Vickerman 1978[1)]	Kinetoplastids	SP	F
Pseudobodo tremulans Griessmann 1913	Bicosoecids	SP	F
Salpingoeca euryoecia Jeuck, Arndt and Nitsche 2014[1) 5)]	Choanoflagellates	P	F
Salpingoeca limnea Carr, Richter and Nitsche[1) 6)]	Choanoflagellates	P	F
Salpingoeca sp. undescribed[1)]	Choanoflagellates	SP	F
Spumella sp. Cienkowski 1870	Chrysomonads	SP	F
Spumella sp. Cienkowski 1870	Chrysomonads	WC	Aliquot PCR
Stagondion pyriformis Carr, Richter and Nitsche [1) 6)]	Choanoflagellates	SP	F
Thaumatomonas coloniensis Wylezich et al. 2007	Thaumatomonads	WC	Aliquot PCR

Abbreviations: P=Pratt, SP=Schmaltz-Pratt,WC=Wright's Chu, [1)] provided by Alexander Mylnikov, [2)] kindly provided by Michael Melkonian, [3)] CCAP 1220/1A, [4)] CCAP 977/2A [5)] description see chapter 2, [6)] descriptions see chapter 4.

Supplement Table 2. Compilation of HF fixation results. Data obtained by literature and own studies.

Organism	Habitat	Fixative	Counting method	Conc. [%]	Hours after fixation	Average % live cell volume	Reference
Monas sp.	m	**G**	C	2.5[1]	2	46.5%	Børsheim and Bratbak (1987)
Monas sp.	m	**G**	F	2.5[1]	2	43.9%	Børsheim and Bratbak (1987)
Monas sp.	m	**G**	P	2.5[1]	2	58.5%	Børsheim and Bratbak (1987)
Monas sp.	m	**bF**	C	30?[1]	2	60%	Børsheim and Bratbak (1987)
Monas sp.	m	**bF**	F	30?[1]	2	50.2%	Børsheim and Bratbak (1987)
Monas sp.	m	**bF**	P	30?[1]	2	57.3%	Børsheim and Bratbak (1987)
Monas sp.	m	**M**	C	(sat.)? [1]	2	49.6%	Børsheim and Bratbak (1987)
Monas sp.	m	**M**	F	(sat.)? [1]	2	33.7%	Børsheim and Bratbak (1987)
Monas sp.	m	**M**	P	(sat.)? [1]	2	86.2%	Børsheim and Bratbak (1987)
Monas sp.	m	**L**	C	-	2	42.9%	Børsheim and Bratbak (1987)
Monas sp.	m	**L**	F	-	2	30.4%	Børsheim and Bratbak (1987)
Monas sp.	m	**L**	P	-	2	65.3%	Børsheim and Bratbak (1987)
Bodo saltans	fw	**M**	I	2.5	-	62%	Chaput and Carrias (2002)
Bodo saltans	fw	**L**	I	1	-	47%	Chaput and Carrias (2002)
Bodo saltans	fw	**G**	I	1	-	36%	Chaput and Carrias (2002)
Bodo saltans	fw	**F**	I	2	-	41%	Chaput and Carrias (2002)
Paraphysomonas imperforata	m	**L**	F	-	-	61.96%	Choi and Stoecker (1989)
Paraphysomonas imperforata	m	**G**	F	1	-	47.16%	Choi and Stoecker (1989)
Paraphysomonas imperforata	m	**G**	F	2	-	42.22%	Choi and Stoecker (1989)

Paraphysomonas imperforata	m	**G**	F	3	-	37.82%	Choi and Stoecker (1989)
Amphidinium carterae	m	**L**	L	2	-	105.3%	Menden-Deuer et al. (2001)
Amphidinium carterae	m	**G**	L	1	-	81.8%	Menden-Deuer et al. (2001)
Amphidinium operculatum	m	**L**	L	2	-	-	Menden-Deuer et al. (2001)
Amphidinium operculatum	m	**G**	L	1	-	78.8%	Menden-Deuer et al. (2001)
Gymnodinium sanguineum	m	**L**	L	2	-	76.6%	Menden-Deuer et al. (2001)
Gymnodinium sanguineum	m	**G**	L	1	-	102.9%	Menden-Deuer et al. (2001)
Gymnodinium simplex	m	**L**	L	2	-	104.3%	Menden-Deuer et al. (2001)
Gymnodinium simplex	m	**G**	L	1	-	85.2%	Menden-Deuer et al. (2001)
Ceratium furca	m	**L**	L	2	-	-	Menden-Deuer et al. (2001)
Ceratium furca	m	**G**	L	1	-	99.9%	Menden-Deuer et al. (2001)
Ceratium fusus	m	**L**	L	2	-	122.6%	Menden-Deuer et al. (2001)
Ceratium fusus	m	**G**	L	1	-	124.4%	Menden-Deuer et al. (2001)
Glenodinium foliaceum	m	**L**	L	2	-	106.2%	Menden-Deuer et al. (2001)
Glenodinium foliaceum	m	**G**	L	1	-	111.4%	Menden-Deuer et al. (2001)
Prorocentrum micans	m	**L**	L	2	-	106.6%	Menden-Deuer et al. (2001)
Prorocentrum micans	m	**G**	L	1	-	116.3%	Menden-Deuer et al. (2001)
Protoperidinium depressum	m	**L**	L	2	-	127.3%	Menden-Deuer et al. (2001)
Protoperidinium depressum	m	**G**	L	1	-	120.6%	Menden-Deuer et al. (2001)
Scrippsiella trochoidea	m	**L**	L	2	-	110.5%	Menden-Deuer et al. (2001)
Scrippsiella trochoidea	m	**G**	L	1	-	118%	Menden-Deuer et al. (2001)
Amphidinium carterae	m	**G**	F	1	-	36.2%	Menden-Deuer et al. (2001)
Bernadinium sp.	m	**G**	F	1	-	66.3%	Menden-Deuer et al. (2001)
Gymnodinium sanguineum	m	**G**	F	1	-	47.9%	Menden-Deuer et al. (2001)
Gymnodinium simplex	m	**G**	F	1	-	31.1%	Menden-Deuer et al. (2001)
Oxyrrhis marina	m	**G**	F	1	-	32.1%	Menden-Deuer et al. (2001)
Alexandrinum catenella	m	**G**	F	1	-	44%	Menden-Deuer et al. (2001)

Ceratium fusus	m	**G**	F	1	-	97.8%	Menden-Deuer et al. (2001)
Ceratocorys horrida	m	**G**	F	1	-	104.5%	Menden-Deuer et al. (2001)
Glenodinium foliaceum	m	**G**	F	1	-	71.6%	Menden-Deuer et al. (2001)
Glenodinium sp.	m	**G**	F	1	-	70.8%	Menden-Deuer et al. (2001)
Prorocentrum micans	m	**G**	F	1	-	94.2%	Menden-Deuer et al. (2001)
Protoperidiniumconicum	m	**G**	F	1	-	101.4%	Menden-Deuer et al. (2001)
Protoperidinium pellucidum	m	**G**	F	1	-	100.2%	Menden-Deuer et al. (2001)
Scrippsiella trochoidea	m	**G**	F	1	-	65.3%	Menden-Deuer et al. (2001)
Entosiphon sulcatum	fw	**G**	L	2	≤ 6	64 ± 9^2	This study
Entosiphon sulcatum	fw	**F**	L	2	≤ 6	125 ± 12^2	This study
Entosiphon sulcatum	fw	**bF**	L	2	≤ 6	106 ± 15^2	This study
Entosiphon sulcatum	fw	**LF**	L	0.5+3	≤ 6	117 ± 15^2	This study
Bodo saltans	fw	**G**	L	2	≤ 6	26 ± 4^2	This study
Bodo saltans	fw	**F**	L	2	≤ 6	22 ± 4^2	This study
Bodo saltans	fw	**bF**	L	2	≤ 6	26 ± 4^2	This study
Bodo saltans	fw	**LF**	L	0.5+3	≤ 6	95 ± 13^2	This study
Procrytobia (=Bodo) sorokini	m	**G**	L	2	≤ 6	50 ± 9^2	This study
Procrytobia (=Bodo) sorokini	m	**F**	L	2	≤ 6	74 ± 12^2	This study
Procrytobia (=Bodo) sorokini	m	**bF**	L	2	≤ 6	63 ± 13^2	This study
Procrytobia (=Bodo) sorokini	m	**LF**	L	0.5+3	≤ 6	64 ± 10^2	This study
Bodo saliens	m	**G**	L	2	≤ 6	58 ± 16^2	This study
Bodo saliens	m	**F**	L	2	≤ 6	67 ± 8^2	This study
Bodo saliens	m	**bF**	L	2	≤ 6	81 ± 14^2	This study
Bodo saliens	m	**LF**	L	0.5+3	≤ 6	86 ± 11^2	This study
Neobodo designis	m	**G**	L	2	≤ 6	62 ± 14^2	This study
Neobodo designis	m	**F**	L	2	≤ 6	73 ± 12^2	This study
Neobodo designis	m	**bF**	L	2	≤ 6	70 ± 14^2	This study
Neobodo designis	m	**LF**	L	0.5+3	≤ 6	97 ± 17^2	This study

Cryptomonas paramecium	fw	**G**	L	2	≤ 6	68 ± 7^2	This study
Cryptomonas paramecium	fw	**F**	L	2	≤ 6	0	This study
Cryptomonas paramecium	fw	**bF**	L	2	≤ 6	0	This study
Cryptomonas paramecium	fw	**LF**	L	0.5+3	≤ 6	85 ± 8^2	This study
Spumella sp.	m	**G**	L	2	≤ 6	71 ± 12^2	This study
Spumella sp.	m	**F**	L	2	≤ 6	65 ± 11^2	This study
Spumella sp.	m	**bF**	L	2	≤ 6	94 ± 15^2	This study
Spumella sp.	m	**LF**	L	0.5+3	≤ 6	89 ± 14^2	This study
Ochromonas sp.	fw	**G**	L	2	≤ 6	38 ± 12^2	This study
Ochromonas sp.	fw	**F**	L	2	≤ 6	29 ± 6^2	This study
Ochromonas sp.	fw	**bF**	L	2	≤ 6	67 ± 15^2	This study
Ochromonas sp.	fw	**LF**	L	0.5+3	≤ 6	72 ± 13^2	This study
Pseudobodo tremulans	m	**G**	L	2	≤ 6	50 ± 11^2	This study
Pseudobodo tremulans	m	**F**	L	2	≤ 6	47 ± 8^2	This study
Pseudobodo tremulans	m	**bF**	L	2	≤ 6	60 ± 8^2	This study
Pseudobodo tremulans	m	**LF**	L	0.5+3	≤ 6	67 ± 11^2	This study
Oxyrrhis marina	m	**G**	L	2	≤ 6	47 ± 9^2	This study
Oxyrrhis marina	m	**F**	L	2	≤ 6	41 ± 7^2	This study
Oxyrrhis marina	m	**bF**	L	2	≤ 6	38 ± 7^2	This study
Oxyrrhis marina	m	**LF**	L	0.5+3	≤ 6	44 ± 8^2	This study
Crypthecodinium cohnii	m	**G**	L	2	≤ 6	68 ± 13^2	This study
Crypthecodinium cohnii	m	**F**	L	2	≤ 6	74 ± 14^2	This study
Crypthecodinium cohnii	m	**bF**	L	2	≤ 6	75 ± 12^2	This study
Crypthecodinium cohnii	m	**LF**	L	0.5+3	≤ 6	85 ± 14^2	This study
Apusomonas proboscidea	fw	**G**	L	2	≤ 6	62 ± 26^2	This study
Apusomonas proboscidea	fw	**F**	L	2	≤ 6	61 ± 38^2	This study
Apusomonas proboscidea	fw	**bF**	L	2	≤ 6	78 ± 26^2	This study
Apusomonas proboscidea	fw	**LF**	L	0.5+3	≤ 6	128 ± 52^2	This study

Paracercomonas crassicauda	fw	**G**	L	2	≤ 6	64 ± 23^2	This study
Paracercomonas crassicauda	fw	**F**	L	2	≤ 6	44 ± 8^2	This study
Paracercomonas crassicauda	fw	**bF**	L	2	≤ 6	56 ± 13^2	This study
Paracercomonas crassicauda	fw	**LF**	L	0.5+3	≤ 6	59 ± 25^2	This study
Cercomonas sp.	fw	**G**	L	2	≤ 6	30 ± 7^2	This study
Cercomonas sp.	fw	**F**	L	2	≤ 6	33 ± 7^2	This study
Cercomonas sp.	fw	**bF**	L	2	≤ 6	35 ± 7^2	This study
Cercomonas sp.	fw	**LF**	L	0.5+3	≤ 6	27 ± 4^2	This study
Percolomonas cosmopolitus	m	**G**	L	2	≤ 6	149 ± 55^2	This study
Percolomonas cosmopolitus	m	**F**	L	2	≤ 6	80 ± 15^2	This study
Percolomonas cosmopolitus	m	**bF**	L	2	≤ 6	68 ± 10^2	This study
Percolomonas cosmopolitus	m	**LF**	L	0.5+3	≤ 6	85 ± 15^2	This study
Codosiga hollandica	fw	**G**	L	2	≤ 6	86 ± 16^2	This study
Codosiga hollandica	fw	**F**	L	2	≤ 6	84 ± 22^2	This study
Codosiga hollandica	fw	**bF**	L	2	≤ 6	244 ± 81^2	This study
Codosiga hollandica	fw	**LF**	L	0.5+3	≤ 6	159 ± 52^2	This study
Salpingoeca euryoecia	fw	**G**	L	2	≤ 6	99 ± 24^2	This study
Salpingoeca euryoecia	fw	**F**	L	2	≤ 6	118 ± 31^2	This study
Salpingoeca euryoecia	fw	**bF**	L	2	≤ 6	141 ± 25^2	This study
Salpingoeca euryoecia	fw	**LF**	L	0.5+3	≤ 6	73 ± 17^2	This study
Salpingoeca limnea	fw	**G**	L	2	≤ 6	62 ± 9^2	This study
Salpingoeca limnea	fw	**F**	L	2	≤ 6	67 ± 13^2	This study
Salpingoeca limnea	fw	**bF**	L	2	≤ 6	105 ± 24^2	This study
Salpingoeca limnea	fw	**LF**	L	0.5+3	≤ 6	103 ± 26^2	This study
Salpingoeca sp.	m	**G**	L	2	≤ 6	107 ± 27^2	This study
Salpingoeca sp.	m	**F**	L	2	≤ 6	153 ± 33^2	This study
Salpingoeca sp.	m	**bF**	L	2	≤ 6	115 ± 23^2	This study
Salpingoeca sp.	m	**LF**	L	0.5+3	≤ 6	171 ± 57^2	This study

Stagondion pyriformis	m	**G**	L	2	≤ 6	138 ± 38[2]	This study
Stagondion pyriformis	m	**F**	L	2	≤ 6	146 ± 37[2]	This study
Stagondion pyriformis	m	**bF**	L	2	≤ 6	138 ± 32[2]	This study
Stagondion pyriformis	m	**LF**	L	0.5+3	≤ 6	161 ± 38[2]	This study

Abbreviations: Habitat: fw: freshwater, m: marine water; Fixatives: **F**=formaldehyde, **G**=glutaraldehyde, **bF**=buffered formaldehyde, **L**=Lugol´s fixative, **LF**=Lugol´s fixative + formaldehyde, **M**= mercuric chloride; counting method: C: Coulter chamber, F: fluorescence, I: inverted microscope + settling chamber, L: light microscopy, P: phase contrast; Conc.: concentration; -: not observed/indicated, sat.: saturated. [1]Børsheim and Bratbak: 1 ml of fixative added to 50 ml sample for glutaraldehyde, formaldehyde, $HgCl_2$, 0.5ml for Lugol.[2] 95% confidence interval, 20-40 organisms measured.

Concluding Remarks and Perspectives

The present work aimed at extending the existing phylogeny and morphology of choanoflagellates, one group of heterotrophic flagellates (HF) with particular interest. To ensure reliable characterization studies on these choanoflagellates and additionally HF in general, methodological tools for identification and quantification have been developed and discussed.

A useful identification tool to unravel the huge diversity of choanoflagellates and HF was still lacking. This problem was faced by developing a short guide to freshwater HF, presented in **chapter 1**. This guide covers all the different morphologies, movement types and feeding modes of commonly occurring HF. It has been tested and improved by several unexperienced and experienced students and scientists and is already used internationally by researchers working with HF. Video sequences have been added to several morphotype descriptions to facilitate the identification; further addition and accomplishment of video sequences is planned and very welcome. It was focused on the commonly occurring HF, but the guide could be widened by the addition of further morphotype/species descriptions. To conclude, it was developed as a summarizing tool for environmental HF and as a contribution to a better understanding of the HF diversity.

Amongst the diversity of HF, special attention was paid to the particular HF group of choanoflagellates (**chapter 2 - 4**). Studying choanoflagellates and especially their ability to form colonies might provide insights into the evolution of multicellularity as they are known to be the closest relatives to Metazoa (e.g. Richter and King 2013). However, the systematics of choanoflagellates with the two orders of Acanthoecida and Craspedida is currently under discussion.

The present dissertation aimed at contributing to this discussed and unresolved classification, especially the classification of the order Craspedida. Several new craspedid species have been isolated and described from world-wide sampling points. Anticipating further taxonomic confusion, most of the genus names described in **chapter 2** were set in quotation marks to indicate that renaming is very likely in future studies when even more sequence data will be available. At present, the genus *Salpingoeca* is showing paraphyletic lineages within the Craspedida. A reappraisal is currently not possible as the type specimen *Salpingoeca gracilis* (described by James-Clark 1867) could not yet be sequenced, so the genus *Salpingoeca* itself lacks a clear classification. The isolation and sequencing of *S. gracilis* might be a future task.

In **chapter 2**, the following species have been described or redescribed by sequencing the 18S and 28S rDNA and using morphological data: *"Salpingoeca" ventriosa* from Greenland (freshwater), *"Salpingoeca" longipes* Kent (1880) from Mallorca (marine), *"Sphaeroeca" leprechaunica* from Ireland (freshwater), *"Salpingoeca" euryoecia* from Uruguay (brackish water), *"Salpingoeca" fusiformis* Kent (1880) from England (freshwater) and *Paramonosiga thecata* from Germany (freshwater). *Paramonosiga* has been established as a new genus. Thus adding these six species has extended the existing craspedid phylogeny. The species description and phylogenetic analysis revealed that the morphological differences in theca shape and the habitat preference of the species (marine, freshwater, brackish water) are – up to now – not mirrored by the phylogeny as the results are clear: No correlation of morphology and/or ecology could be revealed in the phylogenetic analysis. Further sequences are needed to clearly unravel this correlation.

The species addition was extended in **chapter 4**, in which the craspedid phylogeny has been further accomplished by four newly described species and one new genus: *Salpingoeca calixa* from New Zealand (marine), *Salpingoeca limnea* from Greenland (freshwater), *Salpingoeca oahui* from Hawaii (freshwater), *Codosiga hollandica* from

Portugal (freshwater; this species was called "*hollandica"* as it is identical to sequences isolated in the Netherlands) and the new genus *Stagondion pyriformis* from Greece (marine). These species and a set of further choanoflagellates were analyzed within a six-gene analysis. This and the description of several misidentified other species and genera might provide the basis for a complete reorganisation of the choanoflagellate phylogeny in future. Furthermore, evolutionary origins, e.g. wether the last common choanoflagellate ancestor possessed EF-1A and EFL in its genome and the evolution of the different theca morphologies and of freshwater species have been discussed.

A future challenge might be to perform a further six-gene analysis, i.e. to additionally sequence the hsp90, tubA, EF-1A and EFL, of the other newly described species (chapter 2). As the sequencing of choanoflagellates is rather problematic, this additional sequencing of the described species might be very challenging. It was already unsuccessfully tried to sequence the hsp90 and tubA. However, the sequencing might stabilize the 18S and 28S rDNA concatenated phylogeny and provide further evolutionary insights. In addition, later genome and transcriptome studies might complete the molecular studies.

A new group of acanthoecid related choanoflagellates was discovered (**chapter 3)**. This new group was described by the new genus and species *Acanthafallax monosigata*, which was isolated in the River Rhine at Cologne, Germany. *Acanthafallax monosigata* was showing a craspedid morphology (*Monosiga*-like), but the 18S and 28S rDNA analysis showed a relationship to Acanthoecida. In addition, it was forming a phylogenetic cluster together with several uncultured clones. However, these clones have not been described morphologically, but have been sampled in special freshwater or terrestrial habitats including suboxic/anoxic environments. Thus, discovering more species belonging to this group might be very interesting concerning their phylogenetic position and the different habitat preference (suboxic or anoxic conditions) compared to other choanoflagellates. Besides, acanthoecid choanoflagellates are normally occurring in marine habitats, but a transition from marine to freshwater has been documented for a few acanthoecids of the genus

Stephanoeca (Nitsche 2014) and *Acanthocorbis* (Paul 2012)). However, a freshwater related acanthoecid with craspedid morphology has so far and according to textbook knowledge never been observed. Hence, the salinity tolerance and ability of lorica production was tested, but *Acanthafallax monosigata* was neither able to tolerate higher salinities (>1.5 PSU) nor to build a silicified lorica under silica-rich conditions. It is planned to search for silicon transporter (SIT) genes (Marron et al. 2013) in the newly described species to get a deeper insight into the evolution of lorica development. Future growth experiments under suboxic/anoxic conditions might help to clarify whether this species is microaerophilic as most of the other members of the new group are isolated from suboxic/anoxic environments. Furthermore, it would be very insightful to test, whether anoxic tolerance might be important for the evolution of multicellularity, i.e. whether experiments under anoxic conditions might eventually induce the formation of colonies. Currently, colony inducing factors as bacterial sulfonolipids are discussed (Alegado et al. 2012) and testing these triggering factors using choanoflagellates from anoxic habitats would be very interesting. Hence, in general, it would be fruitful to know more about oxygen preferences of choanoflagellates. This would help to get new insights into early animal evolution as Mills et al. (2014) recently conducted oxygen experiments with the seawater demosponge *Halichondria panicea* serving as a model system for earliest metazoans. Mills et al. pointed out that the origin of animals might have not been delayed by low atmospheric oxygen levels as the experiments lead to a high tolerance of suboxic/anoxic condition of the last common ancestor of animals. Performing anoxic experiments with choanoflagellates might thus confirm that the closest unicellular relatives to metazoans might also be microaerophilic and a careful hypothesis that the last common ancestor of choanoflagellates and metazoans might have been able to live under anoxic conditions would be possible.

A summarizing phylogenetic dataset showing all described species is shown in Figure 1. The presented phylogeny of **chapter 2, 3,** and **4** was more or less recovered apart from single relationships: *“Salpingoeca” euryoecia* is now clustering with *“Salpingoeca” fusiformis* with 75% mlBP. The two newly described genera

"*Paramonosiga"* and "*Mylnosiga"* are grouped together but with low mlBP support. The genus names are set in quotation marks as used for the *"Salpingoeca"* species indicating that a future renaming might be very likely.

Besides, isolation and cultivation of choanoflagellates is in general still very challenging. The techniques could be optimized in parts. Observations on salinity tolerance and lorica inducement factors could additionally provide new ecological insights and special feeding modes have been observed. Furthermore, it would be insightful to use the newly described species for studies on sexual processes of choanoflagellates as recent investigations have given hints that sexual reproduction might be possible in choanoflagellates (Carr et al. 2010; Levin and King 2013).

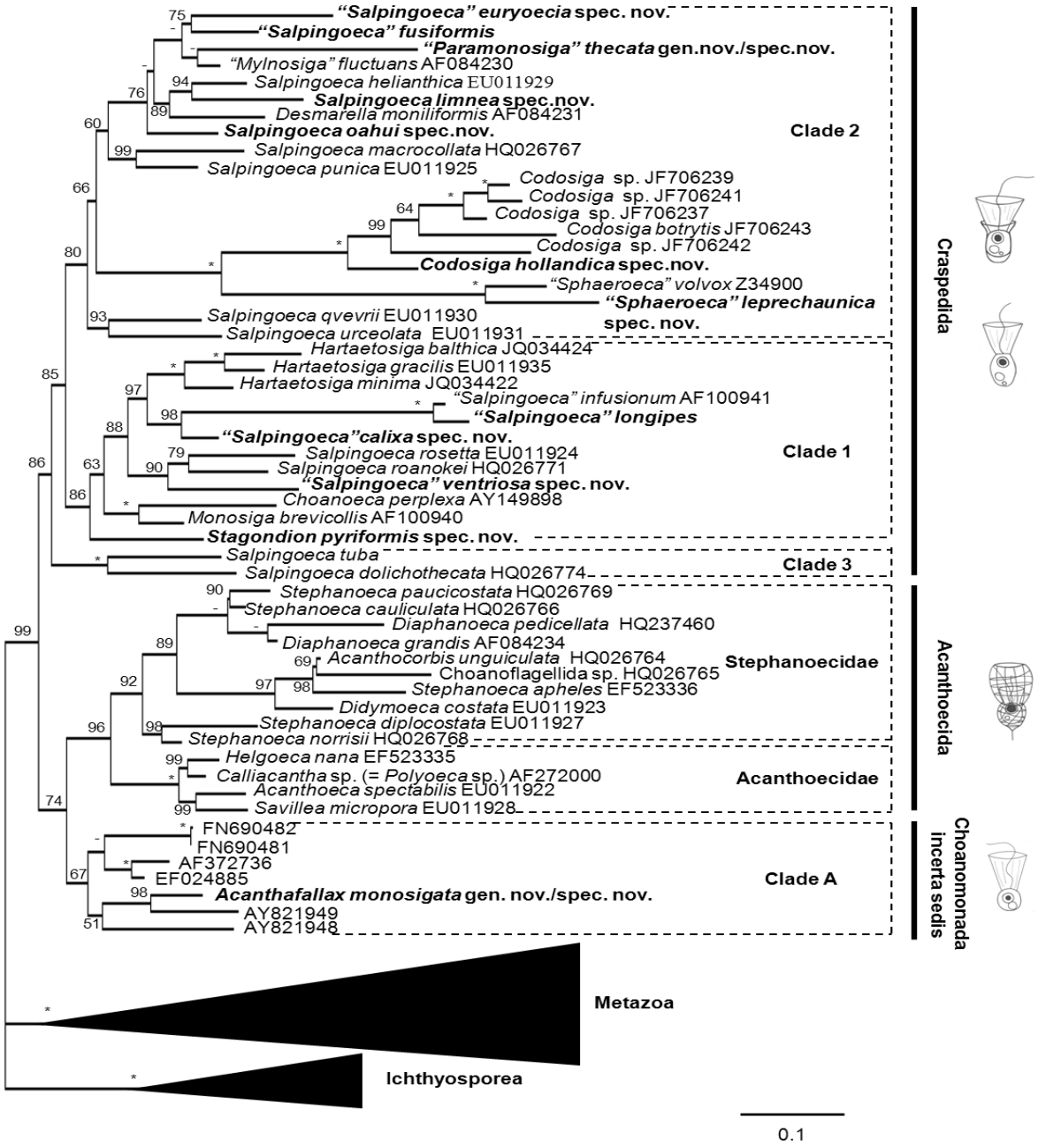

Figure 1. Concatenated maximum likelihood phylogeny of the choanoflagellate sequences (9797 nt) with six-genes: 18S rDNA, 28S rDNA, tubA, hsp90, EFL and EF-1A. The twelve newly sequenced species are marked by bold letters. Support values are offered for RAxML at each node. 100 % RAxML bootstrap percentage support (mlBP) are denoted by *. Support values under 50% mlBP are indicated by a -. The scale bar in the lower right indicates the number of nucleotide substitutions per site. The alignment calculated by Martin Carr (chapter 4) was used as template.

Working with HF in general and choanoflagellates in particular, revealed the need of suitable quantification tools as an accurate quantification of HF in general was not only hampered by the fact that a suitable identification tool was missing but also due to the fact that reliable quantification techniques were lacking. Hence, the present work has been accomplished by a methodological comparison of different HF quantification methods (**chapter 5**). Specific advantages and disadvantages of each method have been opposed. The required method should be chosen carefully with respect to the underlying question. A combination of several methods (at least two methods) might often be most suitable for reliable results (e.g. Auinger et al. 2008; Caron et al. 1989) as each method might be able to close the gap of another.

Taken together, a combination of both morphological and molecular data of choanoflagellates was provided in this dissertation – this was up to now never been carried out in such a complete coverage. The present available dataset on described choanoflagellates (NCBI GenBank) was extended for about one third by adding the newly described choanoflagellates (**chapter 2 - 4**). Besides, the designed short guide to HF offers the basis for the identification of common freshwater HF in general (**chapter 1**). This guide might be used for the live-counting technique, a quantification tool which was compared with other suitable techniques to unravel the high diversity of HF (**chapter 5**).

To sum up, the diversity and systematic view of choanoflagellates, especially the clustering of the different genera of Craspedida etc. is far from being resolved. Thus, this updated systematic view of choanoflagellates might hopefully serve as a further step in the direction towards the highly demanded complete revision of choanoflagellates and hence, in the direction towards evolutionary studies regarding the origin of multicellularity.

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Record of Achievement

Chapter 1 - A short guide to common heterotrophic flagellates of freshwater habitats based on the morphology of living organisms

The design – in form and content - and testing of the taxonomic guide and manuscript draft was exclusively performed by the author of the present dissertation. Parts of the video sequences were recorded and all of them edited by herself. Hartmut Arndt was involved in all technical discussions and discussions regarding the design and has critically read the manuscript.

Chapter 2 – Extended phylogeny of the Craspedida (Choanomonada)

All results described in this chapter – molecular, phylogenetic, taxonomic, and morphologic - were carried out by the author except from the 18S rDNA sequencing data of *“Salpingoeca” fusiformis*, which have been performed by Frank Nitsche as well as most of the sampling and isolation. The complete manuscript draft was provided by the author – Frank Nitsche was involved in data analysis and Frank Nitsche and Hartmut Arndt have critically read the manuscript.

Chapter 3 - A new group of acanthoecid related choanoflagellates from freshwater, sediment and soil

All results described in this chapter – molecular, autecological, phylogenetic, taxonomic, and morphologic - were carried out by the author. *Acanthafallax monosigata* was isolated by Markus Weitere in 1998. The complete manuscript draft was provided by the author – Frank Nitsche was involved in data analysis and Frank Nitsche and Hartmut Arndt have critically read the manuscript.

Chapter 4 - A six-gene phylogeny provides new insights into choanoflagellate evolution

The author was involved in providing most of the sequencing and morphologic data of the five newly described species presented in this chapter - especially for *Codosiga hollandica, Salpingoeca limnea* and *Stagondion pyriformis.* She also co-worked on the manuscript draft and was involved in the taxonomic renaming of species and genera.

Chapter 5 - A comparison of methods to quantify heterotrophic flagellates

The author performed the compilation of data and redesigned all diagrams. She was involved in writing the manuscript draft. The fixation experiments regarding the choanoflagellates have been exclusively carried out by herself.

Teilpublikationen

[1] **Jeuck A, Arndt H** (2013) A short guide to common heterotrophic flagellates of freshwater habitats based on the morphology of living organisms. Protist **164:** 842-860 DOI: 10.1016/j.protis.2013.08.003

[2] **Jeuck A, Arndt H, Nitsche F** (*in press*) Extended phylogeny of the Craspedida (Choanomonada). Eur J Protistol. DOI: 10.1016/j.ejop.2014.06.001

[3] **Jeuck A, Nitsche F, Arndt H** (*under review*) A new group of acanthoecid related choanoflagellates from freshwater, sediment and soil. Protist

[4] **Carr M, Richter DJ, Fozouni P, Jeuck A, Leadbeater BSC, Nitsche, F** *(in preparation)* A six-gene phylogeny provides new insights into choanoflagellate evolution

[5] **Jeuck A, Nitsche F, Domonell A, Wylezich C, Wirth O, Hennemann M, Nopper N, Bergfeld T, Monir S, Scherwaß A, Arndt H** *(in preparation)* A comparison of methods to quantify heterotrophic flagellates

[1] entspricht chapter 1

[2] entspricht chapter 2

[3] entspricht chapter 3

[4] entspricht chapter 4

[5] entspricht chapter 5

Erklärung (gemäß § 4 Abs. (1) Nr.9)

Ich versichere, dass ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit - einschließlich Tabellen, Karten und Abbildungen - , die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie - abgesehen von unten angegebenen Teilpublikationen - noch nicht veröffentlicht worden ist sowie, dass ich eine solche Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen der Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von **Prof. Dr. Hartmut Arndt** betreut worden.

_____

Alexandra Jeuck