UNTERSUCHUNGEN ZUR BIODIVERSITÄT, BIOGEOGRAPHISCHEN VERBREITUNG UND PHYLOGENIE VON CHOANOFLAGELLATEN - UNTER BESONDERER BERÜCKSICHTIGUNG DER POLAREN REGIONEN.

Inaugural-Dissertation

zur

Erlangung des Doktorgrades

der Mathematisch-Naturwisenschaftlichen Fakultät

der Universität zu Köln

vorgelegt von

Frank Nitsche

aus Salzburg

2007

Berichterstatter:

Prof. Dr. H. Arndt

Prof. Dr. E. von Elert

Tag der letzten mündlichen Prüfung:22.06.2007

DANKSAGUNG

- Prof. H. Arndt gilt mein besonderer Dank für die Möglichkeit zur Dissertation, die Betreuung der Arbeit, die vielen hilfreichen Diskussionen und Anregungen und die angenehme Zeit mit ihm als Mensch und Wissenschaftler.
- Meiner Frau möchte ich für die Unterstützung danken, die es mir erst ermöglicht hat, diese Arbeit zu erstellen.
- Claudia Wylezich und Frank Scheckenbach danke ich für Ihre Hilfestellung in molekular-biologischen Fragen.
- Der "Deutschen Forschungsgemeinschaft" (DFG) möchte ich für die im Rahmen dieser Arbeit benötigten Sachmittel und insbesondere für die Finanzierung meiner Stelle durch das geförderte Projekt (**AR 288/8**) danken.
- Herzlicher Dank geht an alle Verwandten, Freunde und Bekannten, die sich bereit erklärt haben, Wasserproben während Ihrer Urlaubs- und Geschäftsreisen zu nehmen und nach Köln zu transportieren. Besonders möchte ich meiner Schwester Sabine Nitsche und Michael Kern für Ihren unentwegten Einsatz in Sachen Probennahme danken.
- Barry Leadbeater danke ich für seine hilfreichen und motivierenden Kommentare.
- Mein ganz besondere Dank gilt meiner Mutter, ohne die diese Arbeit nicht zustande gekommen wäre

INHALTSVERZEICHNIS

EINLEITUNG

In der Protozoologie werden immer wieder drei grundlegende Fragen gestellt, die eng miteinander verbunden sind: Was ist eine Art, wie hoch ist die Artenzahl und wie verbreitet sind Protisten. Und schließlich stellt sich in Verbindung mit Choanoflagellaten noch die Frage nach dem Ursprung der mehrzelligen Lebensformen.

Das biologische Artkonzept nach Mayr (1942) ist bei Protisten, die sich asexuell oder klonal fortpflanzen nicht anwendbar. Mit der Einbindung der Molekularbiologie in die Protozoologie und unter Verwendung der entsprechenden Methoden zeigte sich, dass das Konzept der Morphoart alleine nicht ausreichend ist um die Mannigfaltigkeit der genetischen und ökologischen Variationen innerhalb einer Art wiederzugeben. Dennoch bildet die Morphoart nach wie vor die Grundlage der Taxonomie (Finlay 2004). Vor 50 Jahren wurde der Vorläufer des modernen Artkonzepts durch die Einbezugnahme der asexuellen Fortpflanzung durch Sonneborn (1957) begründet. Danach spricht man beim Vorhandensein einer auch noch so minimalen genetischen irreversiblen Distanz einer neuen Art. Molekularbiologische Untersuchungen der kleinen Untereinheit der ribosomalen DNA innerhalb von einer Morphoart haben eine sehr hohe Variabilität (über 10% p-Distanz) aufgezeigt (Scheckenbach et al. 2006, Massana et al. 2006). Taxonomie sollte daher aus der Bestimmung des Phänotyps, Genotyps und Ökotyps bestehen. Die Methode des genetischen Barcodings (z.B. Tautz et al. 2003) ist hierbei ein hilfreiches Instrument, ebenso wie das Elektronenmikroskop und ökologische Untersuchungen. Doch nur durch Kombination aller Methoden kann in Zukunft eine verlässliche Taxonomie erstellt werden (Will und Rubinoff 2004).

Untrennbar mit dem Artkonzept verbunden ist die Frage nach der Artenzahl. May (1988) hat eine Zusammenstellung der Anzahl beschriebener Arten unter Bezugnahme auf ihre Größe erstellt. Mit abnehmender Größe nimmt die Artenzahl in den logarithmischen Größenklassen wie erwartet zu. Allerdings nimmt die Artenzahl bei einem Schwellenwert von etwa 1mm, unter den die meisten Arten innerhalb der Protisten fallen, wieder ab. Würde man jedoch diese Berechnung extrapolieren, so würde sich bei einer Größe von 1mm eine Artenzahl von 10^6 ergeben und innerhalb der Größenklasse der Protisten eine Artenzahl von 10^8 (Abbildung I).

Derzeit sind 150.000 Protistenarten beschrieben, Schätzungen der Gesamtzahl liegen bei $5 \cdot 10^6$ Arten (Report on the Alfred P. Sloan Foundation Workshop on Protistan Barcoding, 2006). Ein Grund für die möglicherweise zu niedrige Artenzahl von Protisten könnte in dem Mangel an taxonomischen Arbeiten zu finden sein (May

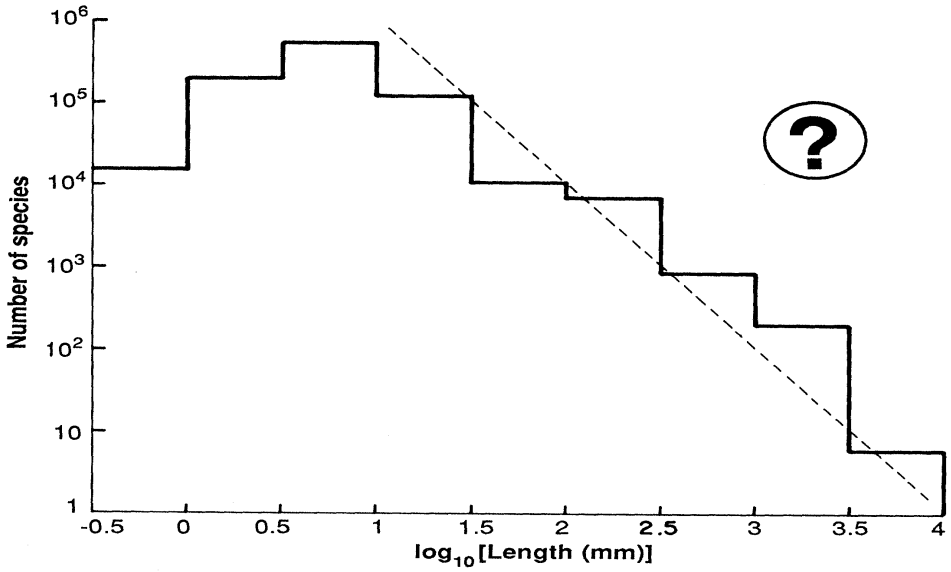

Abbildung I: Schätzung der Artenzahl je Größenklasse (durchgezogene Linie) und des Verhältnisses S=L-2 (S=Artenzahl, L=Länge; gestrichelte Linie). Aus: Robert M. May (1988) „How many species are there on earth" Science **241**: 1441-1449.

1988) sowie in marinen Picoplankton ist eine morphologische Klassifizierung oberhalb des Klassenniveaus nicht möglich (Potter et al. 1997). Innerhalb der Eukaryoten stellen Protisten die individuenreichste Gruppe dar. Heterotrophe Flagellaten treten in Konzentrationen von etwa 10^2 - 10^5 Individuen • ml^{-1} auf (Berninger et al. 1991), Choanoflagellaten zeigen eine Abundanz von bis zu 10^4 Individuen • ml-1 auf (Marchant 2005).

Diese hohe Abundanz, wie sie bei Protisten sehr häufig auftritt zusammen mit der geringen Größe steigert die Wahrscheinlichkeit, dass diese ubiquitär auftritt. Nach Finlay und Fenchels Postulat „alles ist überall" sollte Endemismus bei Protisten nur sehr vereinzelt vorkommen (Abbildung II). Bei entsprechendem Vorhandensein der ökologischen Bedingungen sollte eine Art in der Lage sein, diese Nische weltweit besiedeln zu können. Dies schließt marine wie limnische Gewässer mit ein. Existierender Endemismus wurde bisweilen damit begründet, dass zu wenig Probenstellen untersucht wurden. Dieser Theorie stehen einige Forscher wie der α-Taxonomist Foissner (1999) kritisch gegenüber. Nach Foissner gibt es eine deutliche biogeographische Verteilung, allerdings auch Ausnahmen, die aufgrund ihrer signifikanten Größe und Morphologie in fast jeder Probe gefunden und beschrieben werden, die so genannten Flagship-Species. Neben diesen Arten gibt es aber auch

noch andere Arten, die durchaus eine endemische Lebensweise aufweisen, von Fenchel und Finlay (2004) aber wegen möglichem `undersamplings´ als nichtendemisch bezeichnet werden.

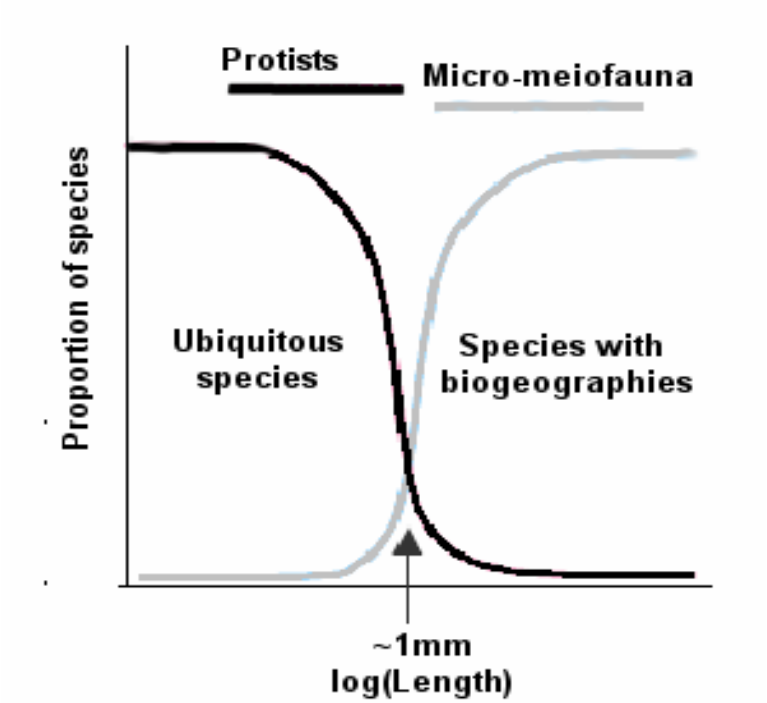

Abbildung II: Schätzung des Artenanteils an ubiquitären und endemischen Arten im Verhältnis zur Größe. Aus Finlay, B.J. (2002) Global Dispersal of Free-Living Microbial Eukaryote Species. *Science* **296**, 1061

Als Gruppe von Organismen wurde für diese Arbeit die Klasse der Choanoflagellaten gewählt. Choanoflagellaten sind einzellige mikrobielle Eukaryoten mit einer globalen Verbreitung, die sowohl in marinen als auch in limnischen Systemen auftreten. Innerhalb der Ordnung der Choanoflagellaten gibt es drei Familien, die Acanthoecidae (Abbildung IIIC), deren Protoplast sich in einer mineralischen Hülle, der Lorica, befindet, und die nur marin oder im Brackwasser vorkommen. Weiters die Salpingoecidae (Abbildung IIIB), deren Protoplast von einer organischen Hülle, der Theka, umgeben wird, sowie die Codonosigidae (Abbildung IIIA), die „nackten" Choanoflagellaten, die weder Theka noch Lorica besitzen und wie die Salpingoecidae sowohl marin bis limnisch verbreitet vorkommen. Hierbei eignen sich besonders die acanthoeciden Choanoflagellaten als Studienobjekt.

Wegen ihrer Lorica, die aus silikathältigen Stäben und Platten besteht, die art-charakteristisch angeordnet sind und damit ein eindeutiges morphologisches Merkmal

darstellen, zählen die Acanthoeciden zu der bestbeschriebenen Familie innerhalb der Choanoflagellaten. Derzeit sind 102 Arten in 30 Genera beschrieben.

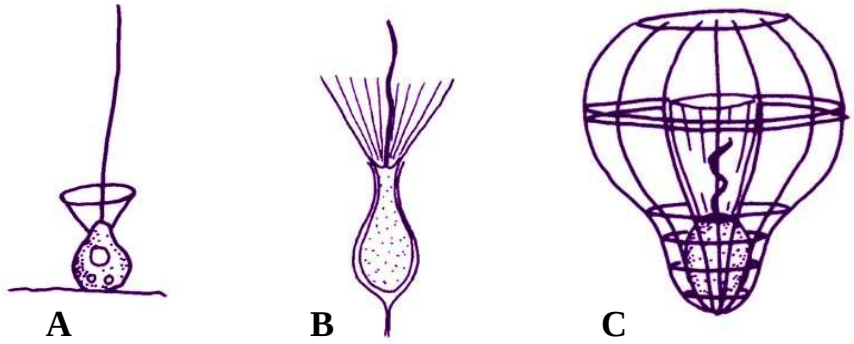

Abbildung III: Schematische Darstellung von typischen Vertretern aus den drei Familien der Choanoflagellaten. A: Codonosigidae; B: Salpingoecidae; C: Acanthoecidae. Aus: Thomsen, H.A. (1992) Loricabærende choanoflagellater (Kraveflagellater). *Plankton in Plankton i de indre danske farvande* (ed. by HA Thomsen), *Havforskning fra Miljøstyrelsen*, **11**,157-194

Zahlreiche Untersuchungen haben gezeigt, dass Protozoen eine bedeutende Position im Stoffumsatz aquatischer Systeme einnehmen (Azam et al., 1983; Güde, 1989; Weisse et al., 1990). Nach dem Konzept des „microbial loop" (Azam et al., 1983) sind die HNF die wichtigsten Prädatoren der Bakterien im Pelagial (Abbildung IV).

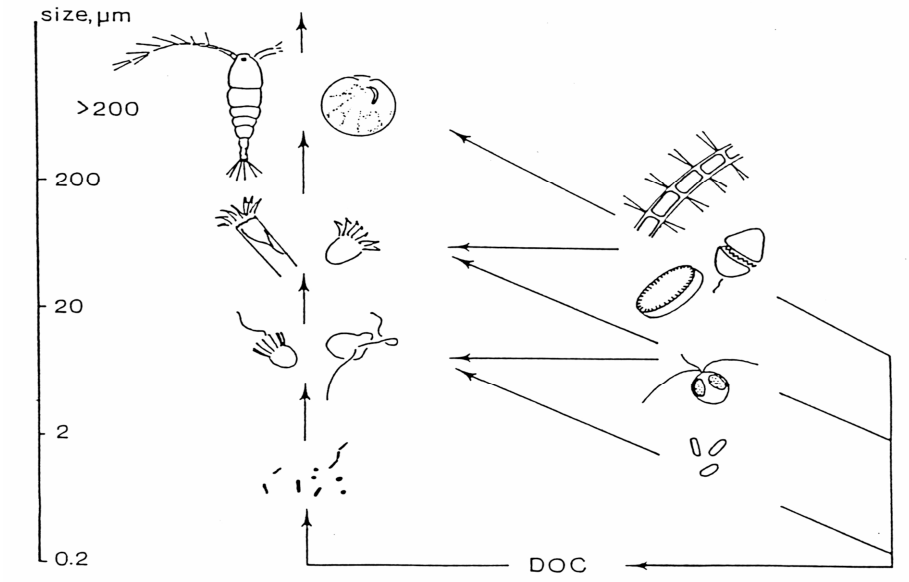

Abbildung IV: Darstellung des mikrobiellen Nahrungsgewebes. Choanoflagellaten sind auf der Ordinate im Bereich von 2-10µm zu finden. Aus: Fenchel, T. (1987) Ecology of Protozoa. The biology of freeliving phagotrophic protists. Science Tech. Publishers, Madison, WI, p 32–52

In Studien zur Artzusammensetzung der Konsumenten von Bakterien findet man Zahlen von 10 bis 90% der Biomasse in Bezug auf Choanoflagellaten. Gerade im wichtigen polaren Nahrungsgewebe stellt diese Ordnung einen sehr hohen Prozentsatz an der Biomasse dar (Pace and Vaqué 1994; Sherr and Sherr 1994).

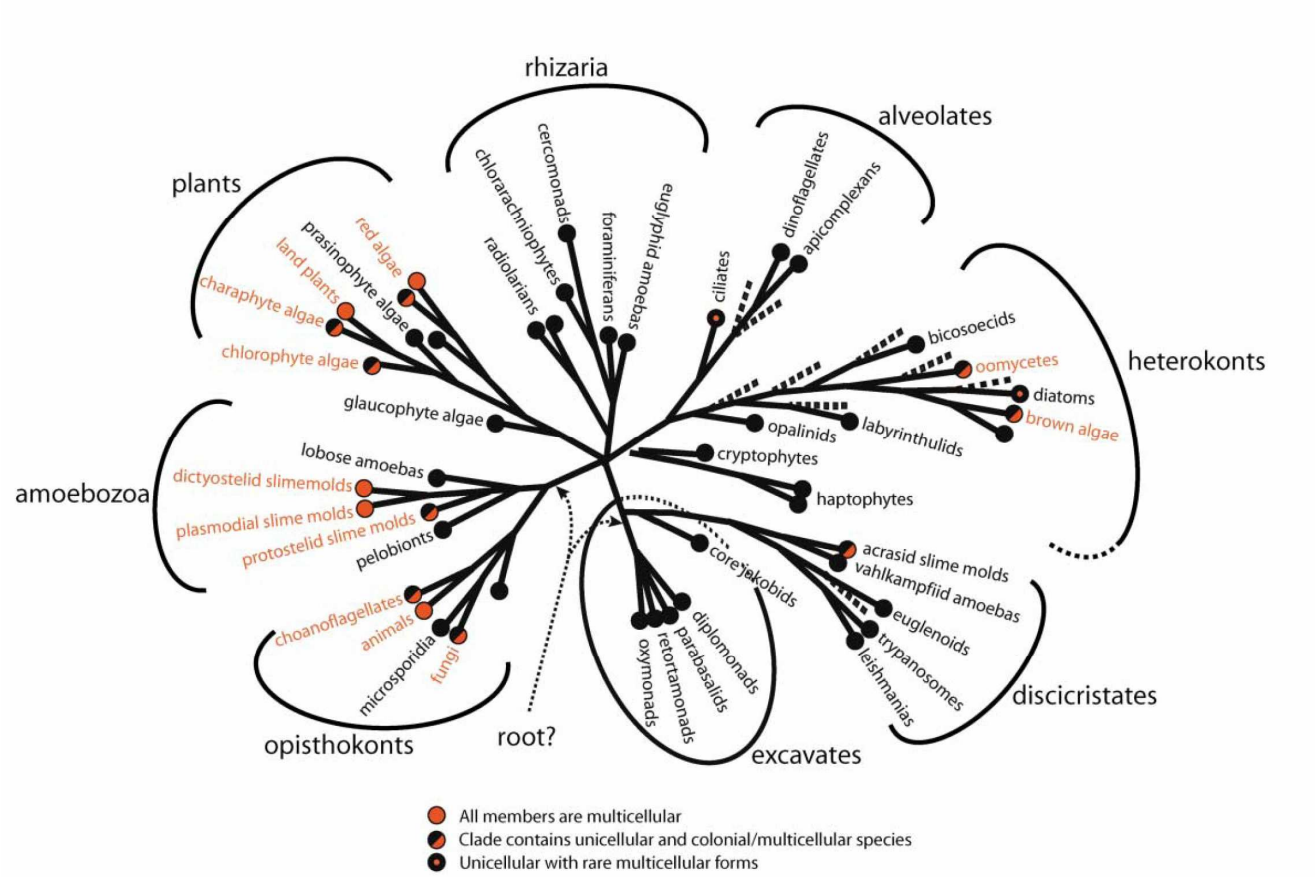

Abbildung V: Phylogenetische Position der Choanoflagellaten innerhalb der Eukaryoten. Aus: Baldauf, S. L. (2003) The deep roots of eukaryotes. *Science*, **300**: 1703-1706

Um einen aussagekräftigen phylogenetischen Stammbaum erstellen zu können, benötigt man ausreichend Sequenzen verschiedener Arten. Zu Beginn dieser Arbeit standen insgesamt neun verschiedene SSU (Small Sub Unit) rRNA Choanoflagellaten Sequenzen aus Gendatenbanken zur Verfügung. Mittlerweile ist dieser Datensatz auf dreizehn verschiedene Sequenzen angewachsen. Lediglich von zwei Choanoflagellatenarten liegt die LSU (Large Sub Unit) rRNA vor. Eines der großen Probleme bei der Isolation von Choanoflagellaten zur Erstellung monoxenischer Kulturen ist ihre Empfindlichkeit gegenüber starken Strömungen. Somit ist eine

Isolierung mittels Pipette bei den meisten Arten annähernd ausgeschlossen. Zudem sind viele Arten benthisch, wodurch die Vereinzelung ebenfalls erschwert wird. Um diese Probleme zu umgehen, wurde für diese Arbeit die Technik der Einzell-PCR (Polymerase Chain Reaction) weiterentwickelt. Mit Hilfe eines Mikromanipulators und einer im Spitzendurchmesser an die zu isolierende Art angepassten Kapillare wurden einzelne Organismen aus dem Medium entnommen und direkt der PCR zugeführt. Die so gewonnene rRNA ist rein klonal und frei von Kontaminationen (bei jeder PCR wurde eine Negativ-Kontrolle durchgeführt).

Die morphologische Eigenheit der acanthoeciden Choanoflagellaten, die Lorica, bietet die einmalige Möglichkeit, die Verbreitung einer Gruppe heterotropher Nanoflagellaten zu untersuchen. Weiters soll in dieser Arbeit am Beispiel von acanthoeciden Choanoflagellaten gezeigt werden, dass die Diversität von Protisten durch ein rein morphologisches Artkonzept möglicherweise unterschätzt wird. Anhand der Untersuchung von bipolaren Choanoflagellatenarten, *Acanthocorbis nana* und *A unguiculata.* sowie der kosmopolitischen Art *Diaphanoeca grandis* wird eine mögliche allopatrische Artbildung untersucht.

Bei der Untersuchung der oben beschriebenen Proben wurden drei neue Arten von Choanoflagellaten gefunden, die ebenfalls in dieser Arbeit beschrieben werden. Diese neuen Arten sowie alle anderen Arten, die im Verlauf dieser Arbeit untersucht worden sind, werden sowohl zur Untersuchung der phylogenetischen Position der Choanoflagellaten innerhalb der Gruppe der Opisthokonta herangezogen als auch für eine Studie der Phylogenie und Taxonomie innerhalb der Choanoflagellaten.

Die Arbeit ist in fünf Kapitel aufgeteilt:

- Kapitel 1 analysiert die biogeographische Verbreitung von acanthoeciden Choanoflagellaten.
- Kapitel 2 beschreibt einen neuen Choanoflagellaten aus dem Genus Diplotheca.
- Kapitel 3 berichtet erstmalig über neue Choanoflagellatenarten aus der Familie der Salpingoecidae aus der Tiefsee.
- Kapitel 4 zieht einen morphologischen und molekularbiologischen Vergleich von Vertretern der bipolar verbreiteten Acanthoeciden (*Acanthocorbis nana* und *A. unguiculata*).
- Kapitel 5 gibt einen Einblick in die phylogenetische Position der Choanoflagellaten anhand des erweiterten Datensatzes an Choanoflagellatensequenzen.

LITERATUR

Azam, F., Fenchel T., Field J. G., Gray J. S., Meyer-Reil L. A., Thingstad F. (1983) The ecological role of the water-column microbes in the sea. *Mar. Ecol. Prog. Ser.* **10**: 257–263.

Baldauf, S. L. (2003) The deep roots of eukaryotes. *Science*, **300**: 1703-1706

Berninger, U. G., Finlay, B. J., Kuuppo-Leinikki, P. (1991) Protozoan control of bacterial abundances in freshwater*. Limnol Oceanogr* **36**: 139–147.

Fenchel, T. (1987) Ecology of Protozoa: The Biology of Free-living Phagotrophic Protists. Science Tech Publishers/Springer Verlag, Madison/Wisconsin, Berlin.

Fenchel, T., Finlay, B. J. (2004) Here and there or everywhere? Response from Fenchel and Finlay. *Bioscience* **54**: 884–885.

Finlay, B. J. (2002) Global dispersal of free-living microbial eukaryote species. *Science*, **296**: 1061-1063.

Finlay, B. J. (2004) Protist taxonomy: an ecological perspective. *Phi.l Trans. R. Soc.Lond., Ser B* **359**: 599–610.

Foissner, W. (1999) Protist Diversity: Estimates of the near-imponderable. *Protist* **150**: 363-368.

Güde, H. (1989) The role of grazing on bacteria in plankton succession, in U. Sommer (ed.) Plankton Ecology: *Succession in Plankton Communities*. Springer-Verlag, Berlin, Heidelberg, New York, pp.337-364.

Marchant, H. J. (2005) Antarctic marine protists. *Choanoflagellates*. (ed. by FJ Scott and H Marchant), pp 326-346. Australian Biological Resources Study, Canberra.

Massana, R., Terrado R., Vovejoy C., Pedros-Alio C. (2006) Distribution and abundance of uncultured heterotrophic flagellates in the world oceans. *Environ. Microbiol*. **8:** 1515-1522.

May, R. M. (1988) How many species are there on earth? *Science,* **247**: 1441-49.

Mayr, E. (1942) Systematics and the Origin of Species. Columbia University Press, New York, USA.

Pace, M. L., Vaqué, D. (1994) The importance of *Daphnia* in determining mortality-rates of protozoans and rotifers in lakes. *Limnol. Oceanogr*. **39**: 985–996.

Potter, D., Lajeunesse, T. C., Saunders, G. W., Anderson, R. A. (1997) Convergent evolution masks extensive biodiversity among marine coccoid picoplankton*. Biodivers. Conserv.* **6**: 99–107.

Scheckenbach, F., Wylezich, C., Mylnikov, A. P., Weitere, M., Arndt, H. (2006) Molecular comparisons of freshwater and marine isolates of the same

morphospecies of heterotrophic flagellates. *Appl. Environ. Microbiol*. **72**: 6638-6643.

Sherr E. B., Sherr, B.F. (1994) Bacterivory and herbivory: key roles of phagotrophicprotists in pelagic food webs, *Microbial Ecology.* **28**: 223-235.

Sonneborn, T. M. (1957) Breeding systems, reproductive methods, and species problems inProtozoa, in Mayr E. (ed.) The species problem. American Association for the Advancement of Science, pp. 155-324.

Tautz. D., Arctander, P., Minelli, A., Thomas, R. H., Vogler, A. P. (2003) A plea for DNA taxonomy. *Trends Ecol. Evol.* **18**:70–74.

Thomsen, H. A. (1992) Loricabærende choanoflagellater (Kraveflagellater). *Plankton in Plankton i de indre danske farvande* (ed. by HA Thomsen), *Havforskning fra Miljøstyrelsen*, **11**:157-194.

Weisse, T., Müller, H., Pinto-Coelho, R.M., Schweizer, A., Springmann, D. (1990) Response of the microbial loop to the phytoplankton spring bloom in a large prealpine lake, *Limnol. Oceanogr*. **35**: 781-794.

Will, K. W., Rubinoff, D. (2004) Myth of the molecule: DNA barcodes for species cannot replace morphology for identification and classification. *Cladistics* **20**: 47–55.

KAPITEL I

Global biogeography of acanthoecid choanoflagellates

ABSTRACT

Aim The unicellular choanoflagellates form an important component of the plankton communities in surface waters of the world ocean. Acanthoecid choanoflagellates possess a lorica (size 5 - 100µm) composed of siliceous costae, a distinct feature which allows the morphological identification of most species. This offers the unique opportunity to study the biogeography of a group of heterotrophic nanoflagellates.

Location The study considered choanoflagellate distribution in the worldwide oceans.

Methods Data on the distribution of Acanthoecidae published in 80 papers within the last 42 years were used to create a taxonomically consistent data set. Analyses were based on the presence/absence of species in biogeographic regions. Clusters were distinguished using Bray-Curtis similarity values.

Results The analysis revealed distinct warm and cold water clusters. Out of 103 acanthoecid species fifteen were recorded until now only in the Arctic/North Atlantic waters and thirteen in the Subantarctic/Antarctic provinces. Nine species showed a bipolar distribution while 54 species were considered as cosmopolitan species. The species richness did not decrease with the latitude.

Main conclusion The data supported the idea that there are epibenthic/coastal species with a restricted geographic distribution. Various regions, especially the open sea, of the world ocean need further sampling and detailed electron microscopical investigations to verify this conclusion.

Key words Ocean biogeography, biodiversity, protists, heterotrophic flagellates, polar regions, species richness

INTRODUCTION

Acanthoecid choanoflagellates play a significant role as major consumers of bacteria in the open ocean, forming the basic transfer of carbon from DOC via bacteria to larger organisms such as ciliates and crustaceans. Heterotrophic flagellates are considered as part of the microbial loop in marine waters (Azam *et al.*, 1983; Gasol & Vaqué, 1993; Arndt, 2000) and have also been found to play a fundamental role in Antarctic waters (e.g. Anderson & Rivkin, 2001).

There is an intensive debate about the distribution patterns of unicellular organisms in literature (e.g. Finlay, 2002; Foissner, 2006). As the fundamental driver of random dispersal is high absolute abundance, and as organism size and abundance are inversely related, there may be some size range where ubiquitous dispersal becomes less likely and where species are more likely to be geographically restricted (Finlay, 2002). This would lead to the conclusion that acanthoecid choanoflagellates, mostly species smaller than 50 µm (protoplast generally smaller than 10 µm), should be ubiquitous or 'common' species. Such 'common' species are characterized by a combination of a broad distribution, an unspecialized habitat and large populations (May, 1988). Acanthoecids characterized by the presence of a species-specific lorica formed by siliceous costae (Leadbeater, 1991) and the limited morphospecies number of only 103 (29 genera) offer the unique opportunity to study the species richness and biogeographic distribution patterns of a family of heterotrophic nanoflagellates.

The only study on the global distribution of choanoflagellates has been carried out by Thomsen (1992). He found several taxa having a cosmopolitan distribution while others appeared to be confined to warm or cold waters. We used recent available data sets and the study of Thomsen (1992) and others for a more detailed analysis of the worldwide distribution of choanoflagellates.

METHODS

The basis of this research were 80 data collections of the last 42 years (see Appendix S2 in Supplementary Material) from all over the world plus our own data collections. The vertical and temporal distribution was not taken into account due to the lack of available data which could be implemented to this study. For consistency only reliable data sources were used. The criteria therefore were light and/or electron

microscopically examinations and the classification down to morphospecies. The dataset was corrected regarding synonyms considering the 'check-list of the marine species' by Brandt (2001) and the webpage of 'micro*scope' (http://starcentral.mbl.edu/microscope/portal.php) to filter out synonyms. Presence/absence data were entered into Microsoft Excel 2003 datasheet (see Appendix S1 in Supplementary Material). To achieve the highest correlation between bioregions and sampling sites the bioregions (after Kelleher *et al.*, 1995) were modified according to the prerequisites of choanoflagellates. The bioregions were defined as variables, the species as cases. Restricted distribution was defined as any species restricted to one bioregion or coherent bioregion temperature cluster (Fig. 2). To be 'common' or ubiquitous the morphospecies had to occur in at least one warm or cold water province on both sides of the equator. Analysis of similarities among species assemblages was conducted using a cluster analysis based on the Bray-Curtis similarity coefficient. Coefficients were calculated with presence/absence-transformed data employing group average sorting (PRIMER version 6). Bioregions which were undersampled (e.g. less then 5 species) were excluded from the analysis and graphical display.

RESULTS

After filtering out all synonyms of the data collection, we established a list of 103 acanthoecid choanoflagellate morphospecies from 29 genera from marine and brackish waters. The survey of data collections showed that especially the Atlantic region is poorly investigated (Fig. 2C). Most sampling sites were close to coastal regions leaving the species composition of open sea mostly uninvestigated. Bearing this in mind and analysing the available data (Fig. 1) we found 13 out of 103 species which were only recorded from the Subantarctic and Antarctica and another 15 species which were present only in samples from the Arctic and Northern temperate provinces. All these 28 morphospecies did not show a bipolar distribution. Compared with these 28 non-bipolar cold-water species, 9 species were found in both polar regions. A total number of 54 out of 103 species were considered to be ubiquitous e.g. they had a transtropical distribution according to their ecological needs (Table 1). Five morphospecies seemed to be restricted to warm-water provinces. For additional 7 acanthoecid species the restricted distribution is most probable, however, the sampling sites were at the boarder to the next bioregion and due to changes of surface currents a distinct assignment was impossible.

Table 1: List of endemic acanthoecid choanoflagellates in different marine provinces

Sub- & Antarctic Region (n=13)	Arctic & Northatlantic (n=15)	Warm water (n=5)
Acanthocorbis prolongata	*Acanthocorbis assymetrica*	*Cosmoeca subulata*
Acanthocorbis tinnabulum	*Conion groenlandicum*	*Diaphanoeca cylindrica*
Acanthocorbis weddellensis	*Monocosta fennica* Thomsen	*Diaphanoeca spiralifurca*
Apheloecion antarctica	*Parvicorbicula aculeatus* Tong	*Diplotheca elongata*
Apheloecion conicoides	*Parvicorbicula manubriata*	*Stephanoeca campanula*
Apheloecion glacialis	*Parvicorbicula pachycostata*	
Calliacantha ankyra	*Parvicorbicula pedunculata*	
Calliacantha frigida	*Parvicorbicula serrulata*	
Cosmoeca takahashii	*Saroeca attenuata*	
Kakoeca antarctica	*Stephanoeca ampulla*	
Parvicorbicula corynocostata	*Stephanoeca cauliculata*	
Parvicorbicula ongulensis	*Stephanoeca constricta*	
Spiraloecion didymocostatum	*Stephanoeca deminutiva*	
	Stephanoeca kentii	
	Stephanoeca pyxidoides	

Cluster analysis revealed a clear separation into a warm and a cold water clusters (Fig. 2A). These clusters could be assigned to physical parameters such as temperature and ocean currents. The cold water cluster contained all cold and cold temperate water provinces except the Subantarctic. All tropical and warm temperate provinces formed the warm water cluster, in which also the Subantarctic was included. Within these two main clusters, five distinct subclusters could be differentiated. The cold water cluster contained a specific Arctic/North Atlantic species community (84% similarity of species community) and a Baltic/Temperate North Atlantic assemblage (83%). The warm water cluster was composed of the temperate Northeast Pacific/tropic North Pacific subcluster (82% similarity), the Northern Indopacific/South Pacific/Mediterranean Sea subcluster (65%), and the tropical North Atlantic/Central Indopacific subcluster (42%). Antarctica as well as the Subantarctic showed a highly specific species community and were separated from the other clusters. We established 21 marine bioregions modified after Kelleher *et al*. (1995) which match the distribution of acanthoecid choanoflagellates, considering the main ocean currents, temperature and sampling sites (Fig. 2B). There was no trend regarding the relationship of species richness of acanthoecid choanoflagellates and the latitude of the different regions (Fig.1).

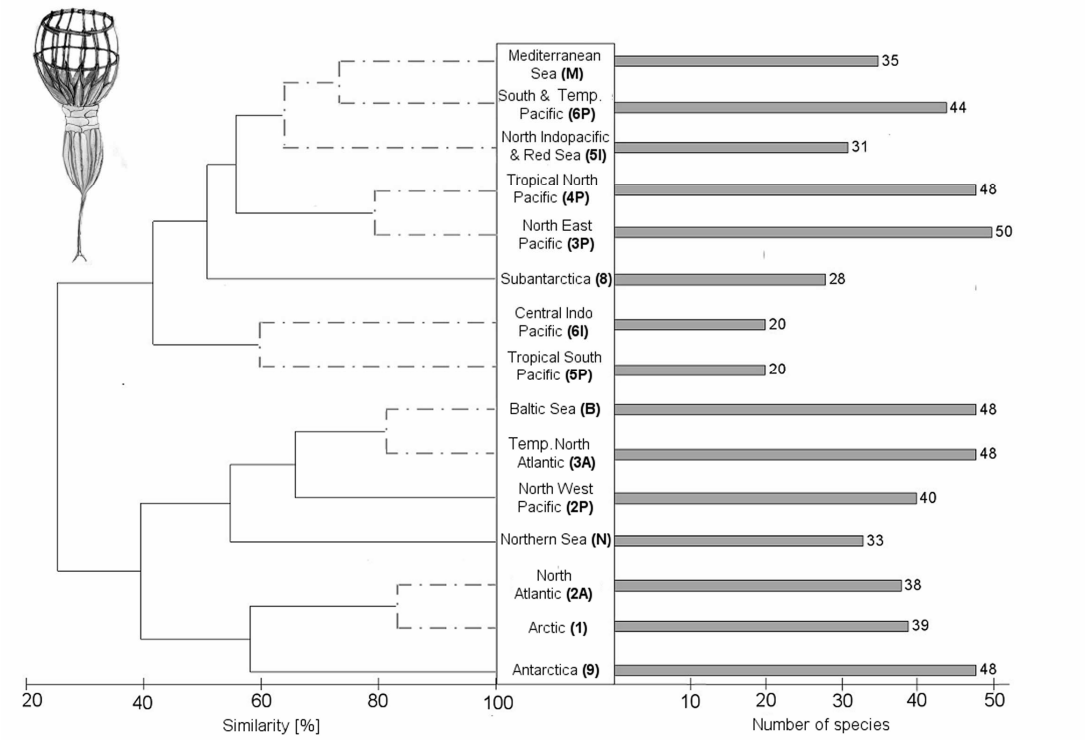

Figure 1: Dendrogram of hierarchical cluster analysis of the worldwide distribution of acanthoecid choanoflagellate communities (left side, with % similarity given in the x-axis) compared with the corresponding number of different morphospecies for each bioregion (right side). The abbreviations of bioregions used for Fig. 2 are given in brackets.

DISCUSSION

Acanthoecid choanoflagellates are one of the few groups of heterotrophic nanoflagellates allowing the study of the geographic distribution. This is due to the unique morphology of their lorica. Though the sampling sites were largely restricted to coastal regions, pelagic species of the open sea were frequently collected. Care had to be taken considering the technical possibilities of researchers to determine morphospecies on the base of their lorica construction. Thus, several older publications had to be left unconsidered for the purpose of global comparisons. The authors are aware of the problematic use of the term "cosmopolitism" in the sense of Fenchel (2005). Despite a cautious interpretation of species records, we found one third of the species to show a restricted biogeo-

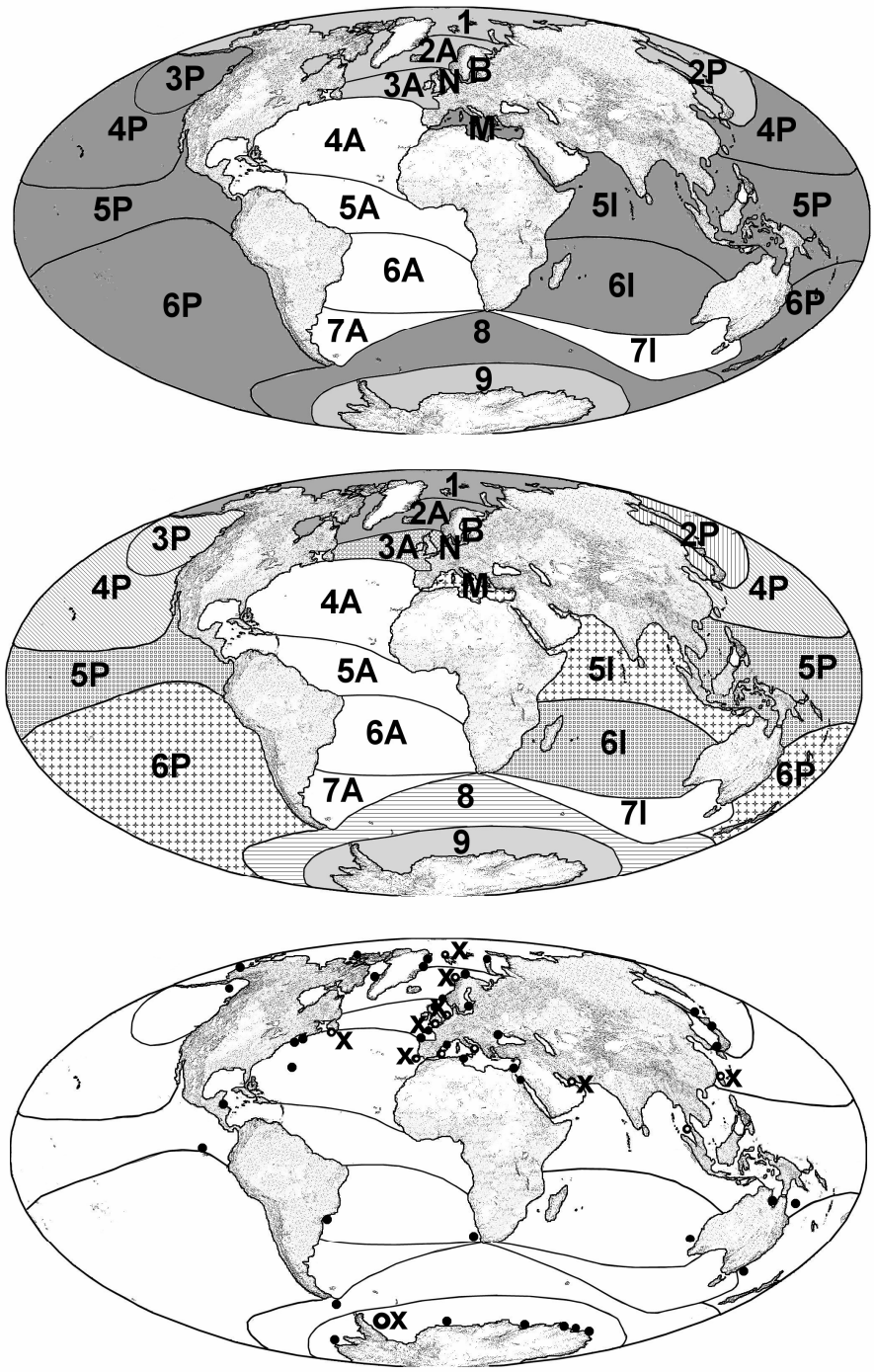

Figure 2: Global distribution patterns of acanthoecid choanoflagellate communities as a result of cluster analysis. Designations of bioregions refer to Fig. 1 A: Warm (dark grey) and coldwater (light grey) clusters; B: Distribution of subclusters; C: Sampling sites of data collections used for the analysis. Dots indicate sampling sites of published data in literature; crosses indicate own samplings (For references and species lists see supplementary material)

graphic distribution compared two third of acanthoecid morphospecies meeting the premise of being cosmopolitans. The unexpected high number of acanthoecid species with a restricted distribution confirms studies on the distribution patterns of ciliates and diatoms (Hillebrand *et al*., 2001). The highest number of species with a presumably restricted distribution was found in polar regions. Abundances of acanthoecid choanoflagellates under the polar ice are several orders of magnitude higher than those from polar pelagic waters (e.g. Hewes *et al*. 1990, Esser 2006). Even if dispersal via cysts in the deep ocean through the conveyor belt takes place, the number of cyst must be tremendously high to amortize the loss during the long transfer. Among choanoflagellates only salpingoecids and codonosigids had been reported from the deep sea (Nitsche *et al*. 2007) making it unlikely that deep sea flows are the major ways of distribution for acanthoecid choanoflagellates. The restricted exchange between choanoflagellate populations of polar regions may have contributed to the speciation process in the two cold water provinces.

For future studies, the analysis of the open sea regions, especially that of the Atlantic, would be desirable for a more complete understanding of acanthoecid distribution patterns. For instance, the clustering of the Subantarctic region in the warm water cluster had to be attributed to the fact that the only available data were recorded from the west wind drift meeting the Peru Current.

Our analysis of literature data included only morphospecies distributions leaving the potentially high number of cryptic species hidden in morphospecies complexes unconsidered. This might even underestimate the number of species with a restricted biogeographic distribution (Darling *et al*., 2002; Scheckenbach *et al*., 2005; Nitsche *et al*., subm.).

ACKNOWLEDGEMENTS

We are greatly indebted to Barry S.C. Leadbeater for valuable discussions. The study was supported by a grant of the German Research Foundation (DFG) to H.A. (AR 288/8 and AR 288/12)

REFERENCES

Anderson, M.R. & Rivkin, R.B. (2001) Seasonal patterns in grazing mortality of bacterioplankton in polar oceans: a bipolar comparison. *Aquatic Microbial Ecology*, **25**, 195-206.

Arndt, H., Dietrich, D., Auer, B., Cleven, E., Gräfenhan, T., Weitere, M. & Mylnikov A.P. (2000) Functional diversity of hetrotrophic flagellates in aquatic ecosystems. *The Flagellates*. (ed. by BSC Leadbeater and JC Green), pp240-268. Taylor & Francis Ltd, London.

Azaam, F., Fenchel, T., Field, J.G., Gray, J.S., Meyer-Reil, L.A. & Thingstad, F. (1983) The ecological role of water-column microbes in the sea. *Marine Ecology Progress Series*, **10**, 257-263.

Brandt, S. (2001) Choanoflagellates. *European register of marine species: a check-list of the marine species in Europe and a bibliography of guides to their identification.*, (ed. by MJ Costello, C Emblow and R White), pp. 57-59. *Collection Patrimoines Naturels,* **50**.

Esser, M. (2005). Doctoral Thesis. University of Cologne.

Fenchel, T. (2005) Cosmopolitan microbes and their "cryptic" species. *Aquatic Microbial Ecology*, **41**, 49-54.

Finlay, B.J. (2002) Global dispersal of free-living microbial eukaryote species. *Science*, **296**, 1061-1063.

Foissner, W. (2006) Biogeography and dispersal of micro-organisms: A review emphasizing protists. *Acta Protozoologica*, **45**, 111-136.

Gasol, J.M. & Vaqué, D. (1993) Lack of coupling between heterotrophic nanoflagellates and bacteria: A general phenomenon across aquatic systems? *Limnology and Oceanography*, **38**, 657-665.

Hewes, C.D., Sakshaug, E., Reid, F.M.H. & Holm-Hansen, O. (1990) Microbial autotrophic and heterotrophic eucaryotes in Antarctic waters: relationships between biomass and chlorophyll, adenosine triphosphate and particulate organic carbon. *Marine Ecology Progress Series*, **63,** 27-35.

Hillebrand, H., Watermann, F., Karez, R. & Berninger, U. (2001) Differences in species richness patterns between unicellular and multicellular organisms. Oecologia, **126,**114–124.

Kelleher, G., Bleakley, C. & Wells, S. (1995) *A Global Representative System of Marine Protected Areas*, The World Bank, Washington, USA.

Leadbeater, B.S.C. (1991) Choanoflagellate organization with special reference to loricate taxa.

Freeliving heterotrophic flagellates. (ed. by DJ Patterson and J Larsen), pp 241–258. Clarendon Press, Oxford.

Marchant, H.J. (2005) Antarctic marine protists. *Choanoflagellates*. (ed. by FJ Scott and H Marchant), pp 326-346. Australian Biological Resources Study, Canberra.

May, R. M. (1988) How many species are there on earth? *Science,* **247**, 1441-49.

Nitsche, F. & Arndt, H (subm) Bipolar comparisons of acanthoecid choanoflagellates.

Nitsche, F., Weitere, M., Scheckenbach, F., Hausmann, K., Wylezich, C. & Arndt, H. (2007) Deep sea records of choanoflagellates with a description of two new species. *Acta Potozoologica*, **46**, in press.

Scheckenbach, F., Wylezich, C., Weitere, M., Hausmann, K. & Arndt H. (2005) Molecular identity of strains of heterotrophic flagellates isolated from surface waters and deep-sea sediments of the South Atlantic based on SSU rDNA. *Aquatic Microbial Ecology*, **38:** 239-247

Thomsen, H.A. (1992) Loricabærende choanoflagellater (Kraveflagellater). *Plankton in Plankton i de indre danske farvande* (ed. by HA Thomsen), *Havforskning fra Miljøstyrelsen*, **11**,157-194

KAPITEL II

A new species of heterotrophic flagellates from Taiwanese brackish waters: Morphological and molecular biological studies of *Diplotheca elongata* nov. spec. and *D. costata* (Choanoflagellida, Acanthoecidae)

Abstract

A new species of acanthoecid choanoflagellate isolated from brackish waters of the Danshui estuary in North Taiwan has a mineralised lorica which consists of two chambers with a total length of 19-36 μm. It shares with *Diplotheca costata* the features of a posterior lorica chamber formed from broad and flattened costal strips and an anterior chamber with spatula-shaped costal strips. The new species has therefore been placed in the same genus and named *Diplotheca elongata*. A phylogenetic analysis of partial SSU of rRNA sequences from *Diplotheca costata* and *D. elongata* supports this taxonomic affiliation. This is a large and distinctive choanoflagellate which has not been reported in any previous study, suggesting that it may be an endemic species of restricted distribution.

Key words. Choanoflagellates, biogeography, phylogeny, biodiversity

Introduction

Protists have been considered to have no restricted biographies as emphasized by a series of articles published by Finlay and Fenchel (e.g. 1999, 2004). Acanthoecid choanoflagellates seem to be a perfect model group within heterotrophic nanoflagellates to study the biogeographic distribution of nanoflagellates due to the species specific morphology of their lorica (Nitsche and Arndt subm.). Choanoflagellates compose between 5 and 40 per cent of nanoflagellate biomass in marine, brackish and freshwater pelagic waters (e.g., Arndt *et al*. 2000). About 200 species of choanoflagellates are known, of which 102 species in 29 genera belong to acanthoecid choanoflagellates, which have mainly been described on the base of the specific shape of the lorica (Leadbeater 1991).

The genus *Diplotheca* belongs to the family Acanthoecidae containing up to now only one species, *Diplotheca costata* described by Valkanov (1970). *D. costata* has been found in the South Pacific Ocean, the North Sea, the Baltic, the Black Sea and the Mediterranean Sea (Valkanov 1970; Thomsen 1992, Tong, 1997). The posterior chamber of the lorica of *Diplotheca* typically consists of broad and flattened costal strips with rounded ends. We isolated a clone of *Diplotheca elongata* from the surface waters of the Chinese Sea in the Taiwan Strait near the city of Danshui, North Taiwan, which represents a new species of this genus. In addition to morphological data, we present also molecular biological studies of SSU rRNA from *Diplotheca elongata* and *D. costata*.

Material and methods

Sampling sites. Material from Taiwan was collected in August 2005 and 2006 from the Taiwan Strait (courtesy of M. Kern). Surface water samples originate from the beach at the estuary of River Danshui near the city of Danshui, North Taiwan (25°10'N/121°26'E). One litre of water was taken in a sterile polyethylene bottle. For *Diplotheca coststa* additional water samples were taken from the coast of the English Channel at Calais, France, in September 2005, and from the coast of the Persian Gulf at Dubai, United Arabic Emirates, in May 2005 and August 2006 (courtesy of S. Nitsche).

The salinity of the samples was about 12 PSU, 34 PSU and 41 PSU in samples from Taiwan, the British Channel, and the Persian Gulf, respectively. Upon arrival of samples in Cologne, the samples were aliquoted to cell culture flasks (50ml) and a sterilized wheat grain was provided as nutrition for autochthonous bacteria

populations as a food source for choanoflagellates. After one week, samples were examined by light microscopy (Zeiss Axiovert 100). Samples containing choanoflagellates were diluted with artificial seawater at the salinity of the sample site. The samples were maintained at 18°C under a 12/12 hour day/night cycle.

Electron microscopy. The preparation of cells basically followed the protocol used by Stoeck *et al.* (2003). The samples were fixed at a ratio of one to one with Bouin's fixative at 4°C for 45 minutes. The fixative contained three parts of saturated with picric acid, one part buffered formaldehyde (38%) and 2% glacial acetic acid, which was added immediately before fixation. To the final solution 0.1 to 0.2% glutaraldehyde (38%) was added. Samples remained in the culture flask and a dehydration series of ethanol with 30%, 50%, 60%, 70%, 80%, 90%, 96% and pure ethanol (each step was done three times and lasted 10 minutes) was carried out. After this procedure, a 50:50 hexamethyldisilazane (HMDS)-ethanol solution was applied for 30 minutes followed by pure HMDS for 30 minutes. Afterwards, the samples were allowed to dry. The bottom of the flasks was cut to appropriate size and stuck to a sample holder. SEM samples were sputtered with a 120Å layer of gold before examination by SEM (Hitachi S-520).

Molecular biology. For single cell PCR, organisms were extracted using a micromanipulator. Cells were transferred to 27µl sterilized water and frozen at -20°C for three hours before PCR. The SSU rDNA fragment was amplified using 18SFor (AACCTGGTTGATCCTGCCAGT) and 28S-D5(rev) (CCGTAGGTGAACCTGCAGAAGGA) primer at a concentration of 1.6 nM followed by a reamplification with the primer pair 82F (GAAACTGCGAATGGCTC) and 18SRev-Ch (CCGTAGGTGAACCTGCAGAAGGA) for SSU. The PCR products were purified using the E.Z.N.A. Cycle-Pure-Kit (Peqlab, Erlangen, Germany). The sequencing of rDNA was done using Big Dye-Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Weiterstadt, Germany) in accordance with the manufacturer's instructions. Primers used for sequencing were 82F, 590F and 18SRev-Ch for SSU. Obtained sequence-parts were manually arranged.

Phylogenetic analyses. Alignments were carried out using ClustalX (Thompson *et al.* 1997) and corrections were made manually with BioEdit. The model (JC69) for maximum likelihood analysis was determined by MrAIC (Nylander 2004) and the ML analysis computed by PhyML (Guindon and Gascuel 2003), using 100 replicates for the bootstrap analysis. Neighbour joining (NJ) was calculated using MEGA 3.1 (Kumar *et al*. 2004) using the JC model and 100 replicates for bootstrap analysis.

Results

Description of *Diplotheca elongata* nov. spec.

Diagnosis: Lorica-bearing protist with the characteristics of its genus (Fig. 1). The lorica of *Diplotheca elongata* is composed of two quite distinct chambers (total length 19-36 µm). The posterior chamber has a length of 5-13 µm and the anterior chamber is between 9 and 15 µm in length (Table 1). The anterior chamber contains 14 to 18 longitudinal costae, each being formed by two costal strips with spatulate ends (Fig. 1A, C). A transverse costa containing five flattened costal strips forms the anterior ring. A second transverse costa is situated in the middle of the anterior chamber (Fig. 1A, B) and is also composed of flattened costal strips. A third ring of costal strips is situated on the base of the spatulated ends of longitudinal strips concealed by the upper part of the posterior chamber (Fig. 1D). The upper part of the posterior lorica chamber is formed by a layer of scale-like flattened strips. The lower part consists of 12 to 16 broad and flattened costal strips with rounded ends. The stalk has a length of 4-8 µm and is built by 3-8 costal strips which are attached to the posterior end of the lorica (Fig. 1D).

Etymology: *elongata* (feminine) from Latin "elongated" in reference to the elongated posterior chamber of the lorica.

Type location: Estuary of the River Danshui near the city of Danshui, North Taiwan (25°10'N/121°26'E).

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

SSU rRNA: Partial SSU rRNA fragments were used for a FASTA search. The result assigned *Diplotheca elongata* into the order of Choanoflagellida, but not into the family of Acanthoecidae (Fig. 3). NCBI accession numbers are: *Diplotheca elongata* EF483922 and *D. costata* EF483923.

Description: The description of *Diplotheca elongata* is based on the observation of cells using light and scanning electron microscopy. Cells are ovoid, with a size range of 8 to 10µm in length and 3.5 to 4 µm in width. The flagellum is 13 to 16 µm long. The collar measures 9 to 15 µm in length. The mineralised lorica of *Diplotheca elongata* consists of two chambers (total length 19-36 µm) (Fig. 1A). The anterior lorica chamber possesses 14 longitudinal costae each composed of two flattened costal strips. The basal coastal strips are spoon shaped (Fig. 1B). In addition to the anterior transverse costa containing five typically flattened costal strips, a second transverse costa which is equally structured is situated in the middle of the anterior lorica chamber (Fig. 1B). The basal posterior lorica chamber is built from 12 to 16 broadly flattened longitudinal costal strips and even broader leaf-like transverse costal strips

form the upper part (Fig. 1D). Inside the lower anterior lorica chamber, a ring composed of crescent strips can be found in individuals, which are going to undergo cell division (Fig. 1E). These are the pre-produced costae for the lorica of the sister cell (Jackson and Leadbeater 1991). The stalk is attached to the posterior end of the lorica by the flattened ends of its costal strips. The number of costal strips forming the stalk ranges from 3-8µm, the length of the stalk is 4-8µm (Fig. 1C).

Table 1. Statistics of lorica measurements of *Diplotheca elongata* (based on 25 individuals) and *D. costata* from the Persian Gulf and the English Channel (based on 20 individuals each). Missing morphological feature are indicated by 'n.p.', not measured values by '-'.

	Total length [µm]	Length of posterior chamber [µm]	Length of anterior chamber [µm]	Width of posterior chamber [µm]	Width of anterior chamber [µm]	Stalk length [µm]
D. elongata						
Average	25.9	9.0	10.9	-	-	6.0
Standard deviation	5.1	2.4	2.1	-	-	1.1
Max.	36	13.5	15	-	-	8
Min.	19.3	5.3	9	-	-	4
D. costata (Persian						
Average	13.0	4.9	8.0	4.7	7.5	n.p
Standard deviation	0.5	0.9	0.3	0.9	1.1	n.p
Max.	15	6	9.5	5	8.5	n.p
Min.	10.5	4	6	4	6	n.p
D. costata (British						n.p
Average	13.0	5	8.0	4.8	7.8	n.p
Standard deviation	1.0	0.4	0.7	0.2	0.7	n.p
Max.	15	6	9	5	9	n.p
Min.	11.5	4.5	6.5	4.5	6	n.p

In addition, we examined two populations of *Diplotheca costata* isolated from the English Channel at Calais (France) and from the coast of the Persian Gulf at Dubai. The morphological appearance (Fig. 2) clearly resembled the original description of Valkanov (1970). Geographical differences could not be detected (Table 1).

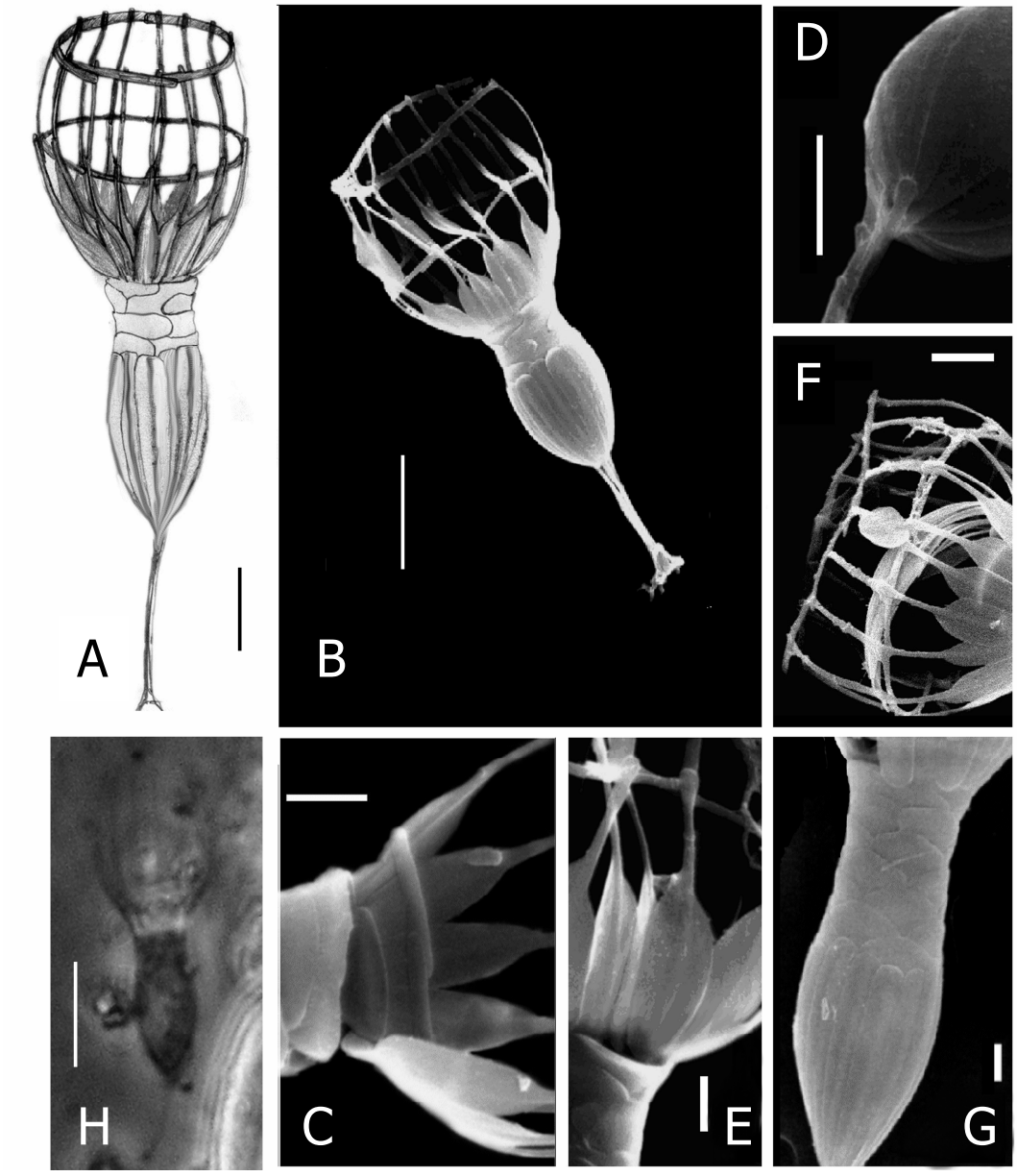

Figure 1. Drawings (A), scanning electron micrographs (B-G) and light microscopic micrographs (H) of *Diplotheca elongata* from the estuary of River Danshui (Taiwan); A – drawing of a specimen; B - complete specimen (scale bars 5 µm); C - detail of costal strips forming the anterior lorica chamber with the typical spatula form; D - close-up of the posterior lorica chamber and the stalk; E – the third transverse costa at the base of the anterior lorica chamber; F – the bundle of crescentic strips inside the lorica; G – elongated upper section of the posterior lorica where the length growth takes place (scale bars 1 µm); H – light microscopical micrograph (scale bar 5 µm).

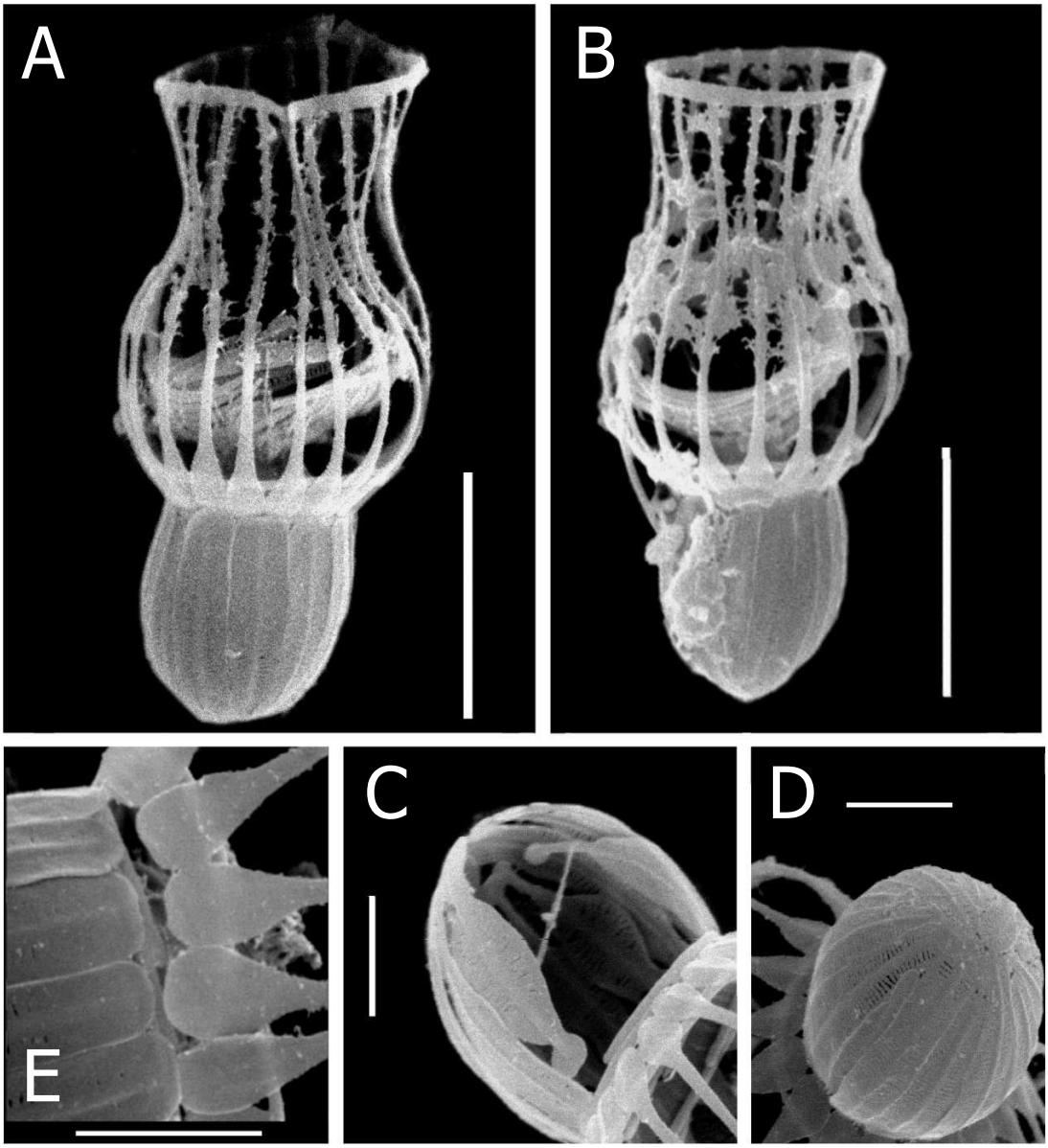

Figure 2 A-F. Scanning electron micrographs of *Diplotheca costata*; A - complete specimen from the British Channel; B - complete specimen from the Persian Gulf; C - the posterior chamber built by flattened costal strips; D - detail of the costal strips from the posterior end; E - detail of the spatula like costal strips forming the anterior lorica (C-E specimens from the Persian Gulf; scale bars A-B 5µm, C-E 2µm)

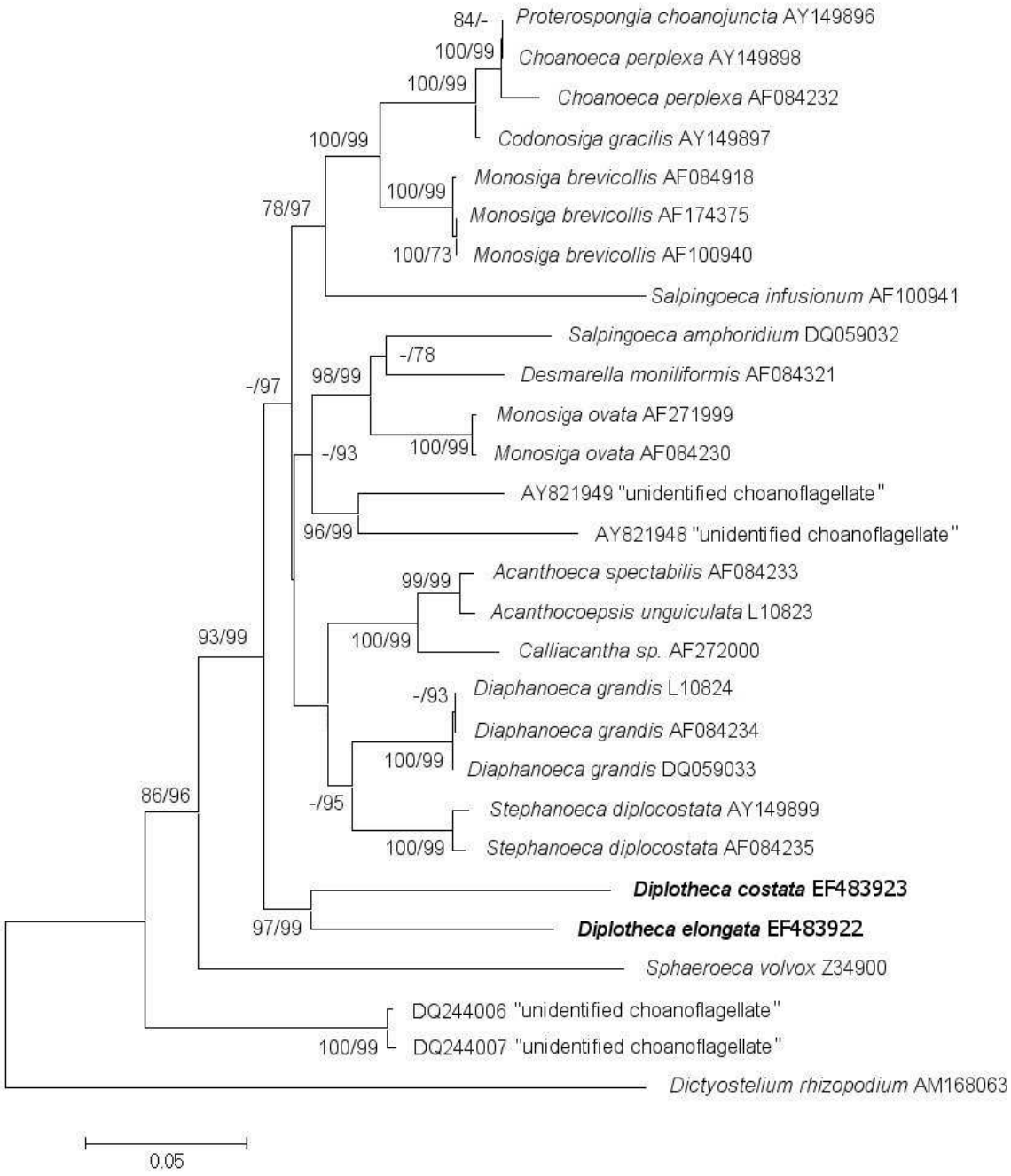

Figure 3 Distance tree of available choanoflagellate sequences based on about 1200 bp long fragments of SSU rDNA using *Dictyostelium rhizopodium* as an outgroup. Bootstrap values represent maximum likelihood values (100 replicates) and neighbour joining (JC) values (100 replicates), values less than 75% are marked '-'. Scale bar marks the genetic distance calculated for the NJ tree.

DISCUSSION

The new species from the Strait of Taiwan, *Diplotheca elongata*, obviously belongs to the same genus as *Diplotheca costata.* The lorica of *Diplotheca elongata* is comprised of on a posterior, an anterior chamber and a stalk. The broad and flattened costal strips which form the posterior chamber and the spatula-shaped costal strips of the anterior lorica chamber are typical for *D. costata* (see Valkanov 1970 and Tong 1997). There is no similarity to any other described acanthoecid choanoflagellate. Due to its size (about 20 to 40µm) this species can be distinguished easily by light microscopy. The biogeographic distribution is so far restricted to the brackish waters of the estuary of River Danshui. Samples from other Taiwanese river estuaries did not contain *Diplotheca elongata*, but it was found in the two following years in different samples from the Danshui estuary. Though this species has very prominent morphological characteristics, it has never been recorded before. It is assumed that this species has a very restricted biogeographic distribution. Considering the fact, that there are about 200 described choanoflagellate species, our recent observations of three new species (Nitsche *et al*. in press and present data) already add 1.5% new taxa to the dataset. Especially in polar regions, endemism seems to be far more common among acanthoecid choanoflagellates than estimated before. Our recent literature survey of more than 120 publications indicated that out of 102 acanthoecid morphospecies 26 were only found either in the Arctic or Antarctic. A total of 32 out of the 102 species were endemic to one biogeographic region (Nitsche and Arndt unpubl.). Considering this the everything-is-everywhere hypothesis seems to be only limited applicable for acanthoecid choanoflagellates.

The analysis of the partial SSU rRNA sequences did not display a clear taxonomic affiliation of both species from the genus *Diplotheca* to the family of Acanthoecidae (Fig. 3). For a more reliable phylogenetic analysis the number of sequenced species must be enlarged. Further studies with *D. elongata* and *D. costata* cultures were not possible as we could not establish continuous cultures. At present, *Diplotheca elongata* together with the sequence of *D. costata* from the Persian Gulf form a cluster out of the family of Acanthoecidae. This is supported by the specific morphology of the costae. There are two other species of acanthoecid-like choanoflagellates which own a similar type of lorica that is also composed of flattened costal strips - *Syndetophyllum pulchellum, Parvicorbicula serrulata* and *Platypleura infundibuliformis* (Leadbeater 1974; Manton and Leadbeater 1975; Thomsen and Boonruang 1983; Thomsen and Moestrup 1983). Future molecular studies of these species will be necessary to show whether these three taxa are related.

Acknowledgements. We thank Barry S.C. Leadbeater and Michael Sleigh for valuable discussions and constructive criticism. This study was financially supported by a grant of the German Research Foundation to H.A. We are very thankful to M. Kern and S. Nitsche for taking and transferring samples from Taiwan and Dubai. For technical support we are indebted to H. Bollhagen, C. Barth, and R. Bieg. Special thanks go to D. Tautz and his Department of Evolutionary Genetics (Institute for Genetics, University of Cologne) for the sequencer facilities.

REFERENCES

Arndt, H., Dietrich, D., Auer, B., Cleven, E., Gräfenhan, T., Weitere, M., Mylnikov A.P., 2000. Functional diversity of heterotrophic flagellates in aquatic ecosystems. In: Leadbeater, B.S.C., Green, J.C. (Eds.), The Flagellates. Taylor & Francis Ltd, London, pp. 240-268.

Fenchel, T., Finlay, B.J., 2004. The ubiquity of small species: Patterns of local and global diversity. BioScience 54, 777-784.

Finlay, B.J., Fenchel, T., 1999. Divergent perspectives on protist species richness. Protist 150, 229-233.

Guindon, S., Gascuel, O., 2003. A simple, fast and accurate algorithm to estimate large phylogenies by Maximum Likelihood. Systems Biology 52, 696-704.

Jackson, S. M., Leadbeater, B. S. C. (1991). Costal strip accumulation and lorica assembly in the marine choanoflagellate *Diplotheca costata* Valkanov. J. Protozool. 38, 97-104.

Kumar, S., Tamura, K., Nei, N., 2004. MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. Briefings in Bioinformatics 5, 50-163.

Leadbeater, B.S.C., 1974. Ultrastructural observations on nanoplankton collected from the coast of Jugoslavia and the Bay of Algiers. J. Mar. Biol. Ass. U. K. 54, 179-196.

Leadbeater, B.S.C., 1991. Choanoflagellate organization with special reference to loricate taxa. In: Patterson, D.J., Larsen, J. (Eds.), Freeliving heterotrophic flagellates. Clarendon Press, Oxford, pp. 241–258.

Nitsche, F., Weitere, M., Scheckenbach, F., Hausmann, K., Wylezich, C., Arndt, H., (2007). Deep sea records of choanoflagellates with a description of two new species. Acta Protozool. 46

Nylander, J.A.A., 2004. MrAIC.pl. Program distributed by the author. Evolutionary Biology Centre, Uppsala University, Uppsala.

Stoeck, T., Fowle, W.H., Epstein, S.S., 2003. Methodology of protistan discovery: from rRNA detection to quality scanning electron microscope images. Appl. Env. Microbiol. 96, 6856-6863.

Thomsen, H.A., 1992. Loricabærende Choanoflagellater (Kraveflagellater). In: Thomsen, H.A. (Ed.), Plankton i de indre danske farvande. Havforskning fra Miljøstyrelsen Vol. 11, Copenhagen, pp. 157-194.

Thomsen, H.A., Boonruang, P., 1983. A microscopical investigation of marine collared flagellates (Choanoflagellida) from the Andaman Sea, S.W. Thailand: species of *Stephanacantha* gen. nov. and *Platypleura* gen. nov. Protistologica 19, 193-214.

Thomsen, H.A., Moestrup, Ø., 1983. Electron microscopical investigations on two loricate choanoflagellates (Choanoflagellida), *Calotheca alata* gen. et. sp. nov. and *Syndetophyllum pulchellum* gen. et comb. nov., from Indo-Pacific localities. Proc. R. Soc. Ser. B 219, 41-52.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Research 24:4876-4882.

Tong, S.M. (1997) Choanoflagellates from Southampton Water, U.K., including the description of three new species. *J. . Mar. Biol. Assoc.,UK,* 77, 929-958.

Valkanov, A., 1970. Beitrag zur Kenntnis der Protozoen des Schwarzen Meeres. Zool. Anz. 184, 241-250.

KAPITEL III

Deep sea records of choanoflagellates with a description of two new species

Abstract

Despite their high abundance and their high importance for the oceanic matter flux, heterotrophic nanoflagellates are only poorly studied in the deep-sea regions. Studies on the choanoflagellate distribution during two deep-sea expeditions, to the South Atlantic (5038 m) and Antarctica (Weddell Sea, 2551 m), revealed the deepest records of choanoflagellates so far. A new species, (*Lagenoeca antarctica*) with a conspicuous spike structure on the theca is described from deep Antarctic waters. *Lagenoeca antarctica* sp. n. is a solitary unstalked free living salpingoecid-like choanoflagellate. The protoplast is surrounded by a typical theca with unique spikes only visible in SEM micrographs. The ovoid cell nearly fills the whole theca and ranges in size from 4 to 6µm. The collar measures 2-3 µm and the flagellum 3-5 µm. A second species, *Salpingoeca abyssalis* sp. n., was isolated from the abyssal plain of the South Atlantic (5038 m depth). Floating and attached forms were observed. The protoplast ranges from to 2 to 4 µm in length and 1 to 2 µm in width. The collar is about the same length as the protoplast and the flagellum has 2 to 2,5x the length of the protoplast. Phylogenetic analyses based on a fragment of SSU rDNA revealed *Salpingoeca abyssalis* to cluster together with a marine isolate of *Salpingoeca infusionum* while *Lagenoeca antarctica* clusters separately from the other codonosigid and salpingoecid taxa. *Salpingoeca abyssalis* and an undetermined *Monosiga* species seems to be the first choanoflagellate species recorded from the abyssal plain.

Key words. Choanoflagellida, deep sea, *Lagenoeca antarctica,* sp. n., *Salpingoeca abyssalis*, phylogeny, SSU rDNA, abyssal plain, Antarctica.

INTRODUCTION

Heterotrophic nanoflagellates belong to the most important bacterial grazers in pelagic and benthic marine ecosystems (Azam *et al.* 1983, Fenchel 1987, Arndt *et al.* 2000). Though the deep-sea floor represents the largest part of the earth's surface, very little is known about its most abundant eukaryotic inhabitants. Previous studies of the deep sea revealed an unexpected diversity of heterotrophic nanofauna (López-García *et al.* 2001, Hausmann *et al.* 2002, Arndt *et al.* 2003, Scheckenbach *et al.* 2005, Hausmann *et al.* 2006). Morphological and molecular biological studies showed that at least some widely distributed nanoflagellates of surface waters can also be found in the deep sea. A variety of protists exists in the deep-sea sediments up to a depth of 5400 m, but only a few species have been recorded up to now. Probably due to their small size (1-10 µm), choanoflagellates from abyssal regions have never been reported. Even molecular biological studies based on clone libraries revealed up to our knowledge no choanoflagellate sequences. This is surprising since choanoflagellates are common in surface marine, brackish and freshwaters forming between 5 to 40 per cent of total nanoflagellate biomass in these habitats (Arndt *et al.* 2000). Patterson *et al.* (1993) have recorded a variety of choanoflagellates from deep plankton and sediment samples indicating their potential occurrence in the deep sea.

About 200 morphospecies of choanoflagellates have been described of which about 50 belong to the family of Salpingoecidae. Members of this family greatly vary in size and shape. All are characterized by the presence of a theca, a layered substructure around the protoplast (Leadbeater 1990). Since recent molecular biological studies have indicated that morphospecies of heterotrophic nanoflagellates are often represented by very different genotypes with sometimes tremendous ecological differences (Scheckenbach *et al.* 2005, 2006; Hausmann *et al*. 2006, Massana *et al.* 2006), we tried to obtain living organisms which could be described on the base of their morphology and SSU rDNA sequence.

MATERIAL AND METHODS

Sampling sites. Material from Antarctica was collected by M. Esser on a cruise of the R/V Polarstern (ANT-XXII/2) in January 2005. Plankton samples were collected by sterilized polyethylene Niskin-bottles from the Weddell Sea from the surface to a depth of 2551 m (63° 46' S and 50° 53' W). The open sampler was washed for more than one hour in the deep sea to minimize the chance of contamination from surface populations. Material from the sediment of the South Atlantic was sampled on a cruise of R/V Meteor (M 63/2) in March 2005. Multicorer samples were taken from the Cape

Abyssal Plain at a depth of 5038 m (28° 7′ S and 7° 21′ E), the Angola Abyssal Plain (9° 56′ S and 0° 54′ E), and the Guinea Abyssal Plain (0° 0′ S and 2° 25′ W). Also here, the open cores were washed for more than one hour in the deep sea to avoid contamination.

On board, samples were aliquoted and species were isolated at normal air pressure and transferred to cell culture flasks (50 ml, Sarstedt). A sterilized wheat grain was provided as nutrition for autochthonous bacteria populations as a food source for choanoflagellates. Each week, samples were examined by light microscopy (Zeiss Axiovert 200 or 40). Samples containing choanoflagellates were registered diluted with artificial seawater of the same salinity. Long-term storage of samples and cultures was carried at 4°C (Weddell Sea samples) or 10°C (abyssal plain samples) in the dark.

Electron microscopy. The preparation of cells basically followed the protocol used by Stoeck *et al.* (2003). Samples were fixed with 50:50 Bouin's fixative mixture (buffered formaldehyde saturated with picric acid and 2% glacial acetic acid added immediately before fixation) with the addition of 0.1 to 0.2% glutaraldehyde, for 45 minutes at 4°C. Samples remained in the culture flask and a dehydration series of ethanol with 30%, 50%, 60%, 70%, 80%, 90%, 96% and pure ethanol (each step was done three times and lasted 10 minutes) was carried out. After this procedure, a 50:50 hexamethyldisilazane (HMDS)-Ethanol solution was applied for 30 minutes followed by pure HMDS for 30 minutes. Afterwards, the samples were allowed to dry. The bottom of the flasks was cut to appropriate size and stuck to a sample holder. SEM samples were sputtered with a 120Å layer of gold before examination by SEM (Hitachi S-520).

Molecular biology. For **s**ingle cell PCR, organisms were extracted using a micromanipulator. Cells were transferred to 27µl sterilized water and frozen at -20°C for three hours before PCR. The SSU rDNA fragment was amplified using 18SFor (AACCTGGTTGATCCTGCCAGT) and 18SRev-Ch (CCGTAGGTGAACCTGCAGAAGGA) primer at a concentration of 1.6 nM followed by a reamplification with the primer pair 82F (GAAACTGCGAATGGCTC) and 18Srev-1 (CGTAACAAGGTTTCCGTAGGT). The PCR products were purified using the E.Z.N.A. Cycle-Pure-Kit (Peqlab, Erlangen, Germany). The sequencing of rDNA was done using Big Dye-Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Weiterstadt, Germany) in accordance with the manufacturer's instructions. Primers used for sequencing were 82F and 590F. Obtained sequences were manually arranged.

Phylogenetic analyses. Alignments were carried out using ClustalX (Thompson *et al.* 1997) and corrections were made manually with BioEdit. The model (GTRG) for maximum likelihood analysis was determined by MrAIC (Nylander 2004) and the ML analysis computed by PhyML (Guindon and Gascuel 2003), using 100 replicates for the bootstrap analysis. Minimum evolution (ME) was calculated using MEGA 3.1 (Kumar *et al.* 2004) using the K2P model and 100 replicates for bootstrap analysis.

RESULTS

Four sampling sites, one pelagic (Weddell Sea) and three benthos samples (South Atlantic) were investigated for the presence of choanoflagellates using live-observation techniques. A salpingoecid choanoflagellate (*Lagenoeca antarctica*) could be isolated and cultivated from the Antarctic pelagial (2551 m depth) and grown at 4°C. From the South Atlantic stations, deep-sea cultures of choanoflagellates could be obtained from the Cape Basin (5034-5084 m depth) and from the Guinea Basin (5063-5066 m depth). At both stations, the new species *Salpingoeca abyssalis* as well as an undefined *Monosiga* species were found. No choanoflagellates could be recorded from the Angola Basin (station at a depth of 5650 m). Unfortunately, *Monosiga* sp. did not grow in culture and we were not able to carry out detailed morphological and molecular biological studies with this species.

Rough abundance estimates using the liquid aliquot method (Butler and Rogerson 1995) revealed choanoflagellate densities in the deep sea lower than 1 ind./l in the pelagial of the Weddell Sea as well as in the bottom waters of the South Atlantic. Both choanoflagellate species of the deep sediments were not only found in the sediment surface layers (shells of foraminiferans) but also on small stones and shells of invertebrates. Remarkable was the slow beating rate of the flagella of the deep-sea choanoflagellates compared to choanoflagellates which we isolated from surface waters when cultivated under similar conditions.

***Lagenoeca antarctica* sp. n.**

Diagnosis: Unstalked choanoflagellate with the characteristics of salpingoecids (Figs. 1A-E). The species specific characteristics of *Lagenoeca antarctica* are small thorn-like elevations on its theca visible by SEM. The size of the ovoid protoplast which fills the theca completely ranges from 4 to 6 µm in length and 2 to 3 µm in width. The collar measures 2-3 µm and the flagellum is 3-5 µm in length. The cells are found free-swimming using the flagellum for propulsion.

Etymology: *antarctica* in reference to the sampling region.

Type location: Bathypelagial of the Weddell Sea in Antarctica (63° 45,7' S 50° 53,2'W) at a depth of 2551 m.

Holotype: The illustration of the specimen in Fig. 3.1A.

Taxonomic position: Although the morphological studies, especially the existence of a theca, support an affiliation of the new species to the Salpingoecidae, the phylogenetic examinations of the partial SSU rDNA (Fig. 3) indicate a significant phylogenetic distance of the species from other choanoflagellate taxa and do not allow yet a distinct assignment to the family of Salpingoecidae.

Description: The description of *Lagenoeca antarctica* sp. n. is based on the observation of cells using scanning electron microscopy and light microscopy. The protoplast is ovoid in shape and fills nearly the whole theca. The length of the protoplast ranges from 4-6 µm in length and from to 2-3 µm in width (Tab. 1). The theca is covered with spike or thorn like elevations only visible in EM (Figs 1B-E), which are irregularly spread over the whole surface. The relatively short flagellum (3-5 µm, Figs. 1B, C) emerges from a short collar (2-3 µm). The new choanoflagellate species appears only free swimming in cultures.

***Salpingoeca abyssalis* sp. n.**

Diagnosis: Small choanoflagellate possessing a theca. Benthic forms are attached by a stalk to the substratum while free-swimming forms are unstalked (Figs 2A-G). Protoplasts are oval in shape and covered by a theca. The size of the protoplast ranges from 1-2 µm in width and 2-4 µm in length. The flagellum has a length of 2 to 2.5x the length of the protoplast, while the length of the collar is similar to the length of the protoplast.

Etymology: *abyssalis* in reference to the sampling site in the abyssal.

Type location: Cape Abyssal Plain (South Atlantic) at a depth of 5038 m (28° 6.7' S and 7° 20.8' E).

Holotype: The illustration of the specimen in Figs. 2 A, B.

Taxonomic position: According to the phylogenetic studies of the partial SSU rDNA (Fig. 3), *Salpingoeca abyssalis* clusters together with *Salpingoeca infusionum*, but not the other salpingoecids.

Description: The description of *Salpingoeca abyssalis* sp. n. is based on the observation of cells using scanning electron microscopy and light microscopy. The protoplast is ovoid shaped and surrounded by a theca which it fills completely (Figs. 2 B, C). The size of the cell ranges from 2 to 4 µm in length and 1 to 2 µm in width. The quite long flagellum measures 4-10 µm (Fig. 2D), the collar is 2 to 4 µm long (Tab. 1).

S. abyssalis was found free-swimming (theca without a stalk) but also frequently attached to the substratum (theca with a stalk). Both forms are illustrated in Figure 2.

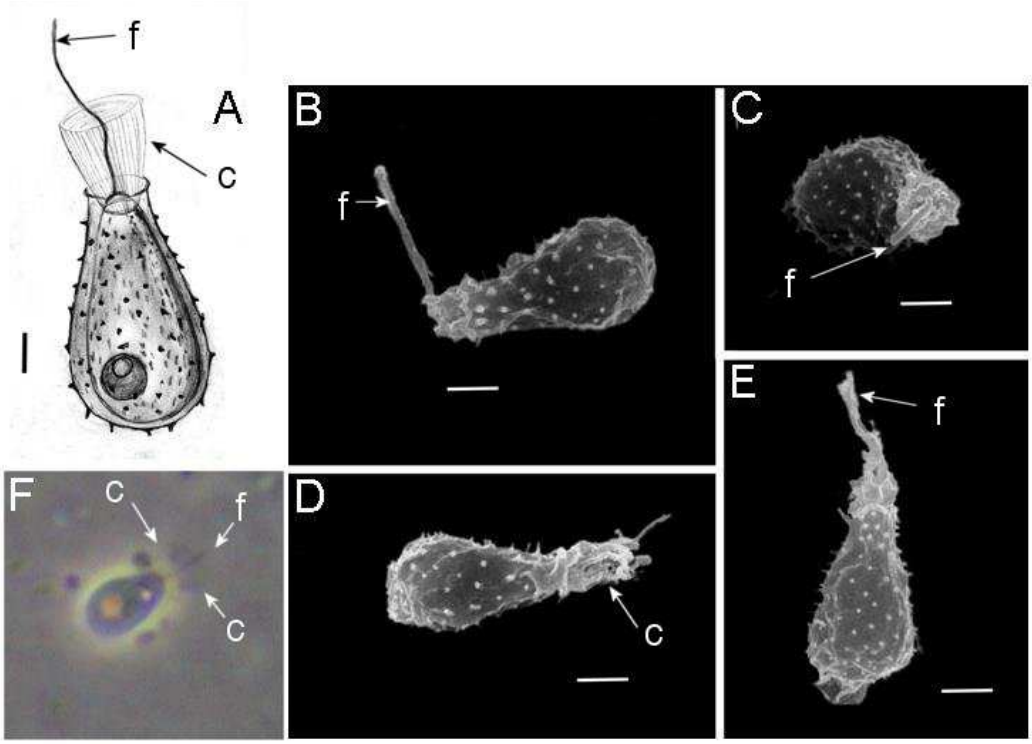

Figure 1 A-F: *Lagenoeca antarctica* sp. n. A: Drawing. B–E: Scanning electron micrographs (B: complete specimen; C: close-up of collar and flagellum; D and E: close-up of the spiked structure on the theka; scale bars 1 µm). F: Light microscopical micrograph; A-F: c=collar, f=flagellum.

Table 1: Statistics of size measurements of *Lagenoeca antarctica* sp. n. and *Salpingoeca. abyssalis* sp. n. (floating form) (n=25).

	Protoplast		Collar	Flagellum
	Length [µm]	Width [µm]	Length [µm]	Length [µm]
L. antarctica				
Average	5.05	2.43	2.47	4.03
Standard **D**eviation	0.76	0.41	0.39	0.81
Max.	6	3	3	5
Min.	4	2	2	3
S. abyssalis				
Average	2.63	1.28	2.52	5.54
Standard **D**eviation	0.68	0.35	0.66	1.53
Max.	4	2	4	10
Min.	2	1	2	4

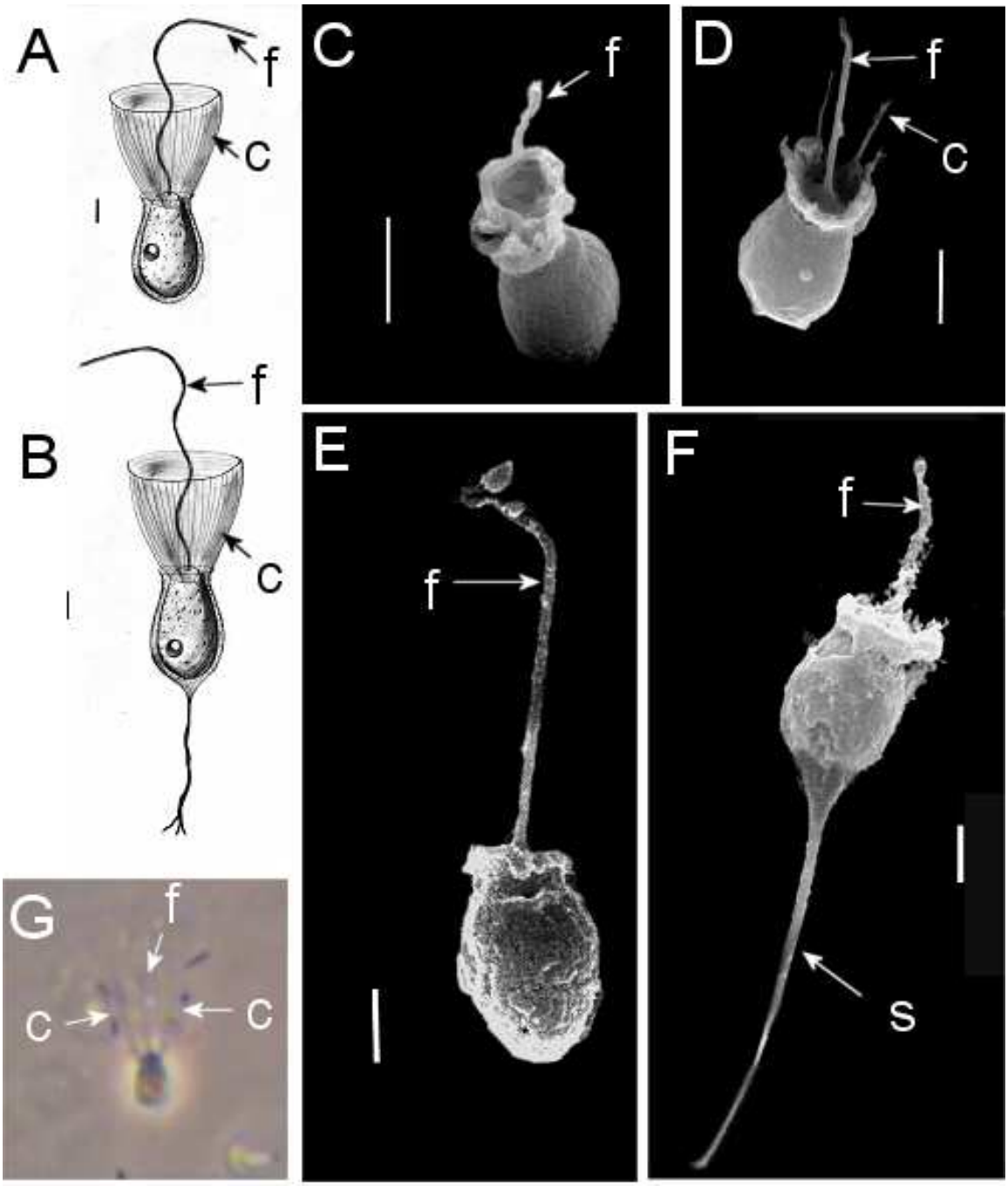

Figure 2 A-G: *Salpingoeca abyssalis* sp. n. A-B: Drawings (A: floating form, B: attached form). C-F: Scanning electron micrographs (C and D: close-up of the collar; E: complete floating specimen with intact flagellum; F: complete attached specimen with a stalk; scale bars 1 µm). G: Light microscopical micrograph of the floating form; A-G: c=collar, f=flagellum, s=stalk.

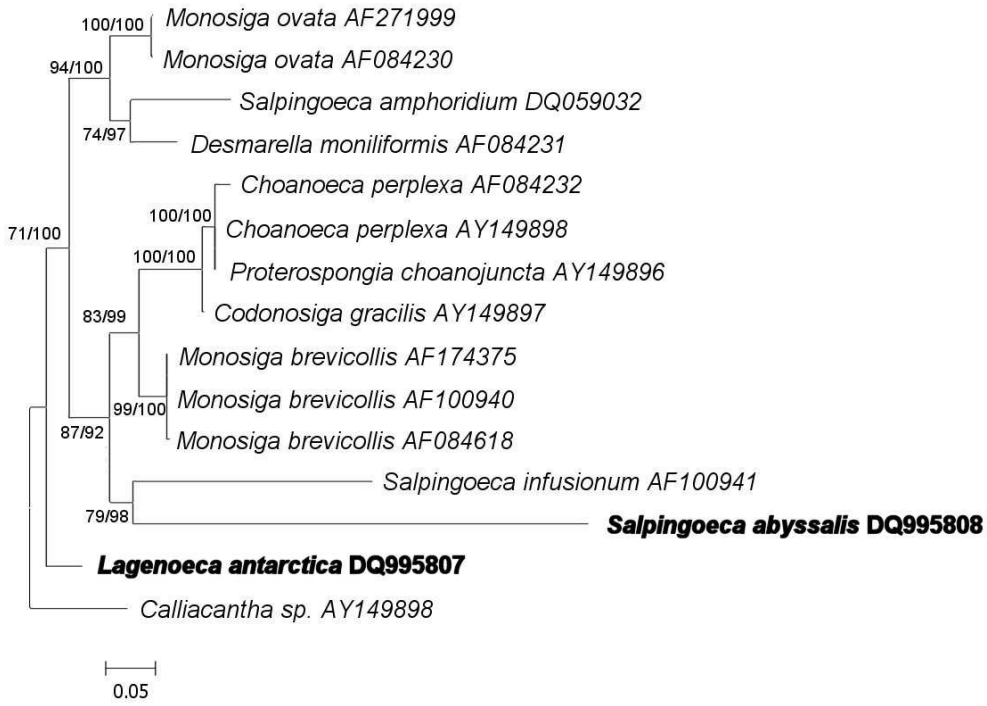

Figure 3: Distance tree of codonosigid and salpingoecid choanoflagellates based on about 1000 bp long fragments of SSU rDNA using an acanthoecid as an outgroup. Bootstrap values represent maximum likelihood values (100 replicates) and minimum evolution values (100 replicates).

The phylogenetic position of both species based on the SSU rDNA published in Genbank identified *Salpingoeca abyssalis* to cluster together with a marine isolate of *Salpingoeca infusionum* while the new species *Lagenoeca antarctica* clustered separately from the other codonosigid and salpingoecid taxa (Fig. 3). Acanthoecid taxa were chosen as an outgroup since this family forms a distinct cluster within the choanoflagellates.

DISCUSSION

Choanoflagellates should be considered as typical members of the deep-sea protistan fauna. Our records from the deep Atlantic abyssal plains are more than 2500m deeper than earlier findings of choanoflagellates (e.g. Patterson *et al.* 1993, Atkins *et al.* 2000). There is surprisingly little information about the community structure of heterotrophic flagellates from the deep sea. Except for cultivation studies there is nearly nothing known about the composition of heterotrophic flagellates with resolution down to the level of genera or species in the greatest biotope of the earth.

The cultivation of samples from the deep-sea floor and pelagic habitats revealed that several taxonomic groups of heterotrophic flagellates (at least cysts) can occur even in very deep parts of the water column (Patterson *et al.* 1993, Atkins *et al.* 2000, Hausmann *et al*. 2002, Arndt *et al.* 2003). Though isolates from the Cape and Guinea Abyssal Plain might originate from viable (!) cysts only, the discovery of *Lagenoeca antarctica* from the deep Antarctic waters with its very characteristic structure of the theca which has never been reported from the well-investigated Antarctic surface waters might point to the existence of a specific deep sea choanoflagellate community. Although, a contamination of samplers could not completely be excluded, the chance of contamination is extremely low. Never any phototroph species, which occurred in high concentrations in overlaying surface waters, appeared in the samplers (neither Niskin bottles nor core samples). Choanoflagellate concentrations in surface waters are more than three orders of magnitude lower than bacteria for which butterfly samplers have been proven to be a useful alternative to avoid contamination. Intensive rinsing of open samplers for more than one hour in the deep sea was done to minimize the chance of contamination from surface populations. The dilution of a possible contamination from surface waters is about 10^7 - 10^8 to 1 giving an idea how low the chance of contamination was. Furthermore, parallel cultivations of surface water samples revealed no representatives of the described species.

While choanoflagellates may occur in concentrations of up to 1000 ind./ml in productive surface waters, their abundances are probably several orders of magnitude lower in the deep sea. This is due to the low flux of organic matter. The biomass of deep benthic communities is typically only 0.001-1% of that in shallow-water communities (Smith and Demopoulos 2003). Low food concentrations together with low temperatures yield relatively low rates of growth, respiration, reproduction, recruitment and bioturbation in the deep sea (Gage and Tyler 1991, Smith and Demopoulos 2003).

Both described species differ clearly from other previously described choanoflagellates with regard to their morphology and phylotype. The EM micrographs of *Lagenoeca antarctica* show that the theca possesses unique spike-like structures on its surface, invisible by light microscopy. The biological significance of this structure is unclear, but it was found on all specimens. *L. antarctica* was isolated from the pelagial and found only free-swimming in cultures and has most probably to be considered as a planktonic species. The only described choanoflagellates which are more or less resembling *L. antarctica* in shape are *Lagenoeca cuspidata* Kent 1880 and *L. variabilis* Skuja 1956 (Kent 1880, Skuja 1956, Zhukov 1993). However, *L. cuspidate* and *L.*

variabilis are freshwater species. In the description of both species the authors described a "granulated" structure in the theca visible by light microscopy. Skuja (1956) described the granulated structure as detritus particles which adhere to the colloidal theca. *L. antarctica* does not show this structure when observed by light microscopy. In EM studies this morphological characteristic resembled more some type of spinous projections than granules. The granulated structures are not detritus particle but a structure of the theca itself. Probably, the new species is a member of a new genus, however, molecular biological studies of other members of this genus are necessary to prove this. Since the recorded *L. antarctica* showed the highest similarity to *L. variabilis* on the light microscopic level, the recorded new species was assigned to this genus.

Salpingoeca abyssalis is a very tiny salpingoecidae with a length of the protoplast of only 2 to 4 µm. The flagellum of *S. abyssalis* is - compared to other choanoflagellates (incl*. S. marina*) - quite long. The theca does not show any special structures. Both, free-swimming unstalked and attached stalked forms were frequently found in cultures. The occurrence of unstalked forms may aid in the dispersal of the population. The highest similarity to this new species is given by *S. marina* James-Clark 1867, but this species is significantly larger and is at least double in size to that of *S. abyssalis*. The ratio of the length of the flagellum to the length of the protoplast is about 1:2 in *S. abyssalis* compared to 1:1.5 in *S. marina* (James-Clark 1867)

The SSU rDNA placed the strain of *Lagenoeca antarctica* with its characteristic theca out of the cluster of salpingoecidae (Fig. 3.3). There seems to be evidence that salpingoecid and codonosigid choanoflagellates are polyphyletic groups and have to be reassigned in near future. More sequences of related species are necessary to resolve the phylogeny of choanoflagellates.

Acknowledgements. This study was financially supported by grants of the German Research Foundation to H. A. and K. H. We are very thankful to M. Esser for taking and transferring samples from Antarctica. We are indebted to B. S. C. Leadbeater for critical reading the manuscript und his helpful comments. For technical support we thank H. Bollhagen, C. Barth, and R. Bieg. Special thanks go to D. Tautz and his Department of Evolutionary Genetics (Institute for Genetics, University of Cologne) for the sequencer facilities.

REFERENCES

Arndt H., Hausmann K., Wolf M. (2003) Deep-sea heterotrophic nanoflagellates of the Eastern Mediterranean Sea: qualitative and quantitative aspects of their pelagic and benthic occurrence. Mar. Ecol. Prog. Ser. **256**: 45-56

Arndt H., Dietrich D., Auer B., Cleven E., Gräfenhan T., Weitere M., Mylnikov A. P. (2000) Functional diversity of heterotrophic flagellates in aquatic ecosystems. In: The Flagellates, (Eds. B. S. C. Leadbeater, J. C. Green) Taylor & Francis Ltd, London, 240-268

Atkins M. S., Teske A. P., Anderson O. R. (2000) A survey of flagellate diversity at four deep-sea hydrothermal vents in the Eastern Pacific Ocean using structural and molecular approaches. J. Eukaryot. Microbiol. **47:** 400-411

Azam F., Fenchel T., Field J. G., Gray J. S., Meyer-Reil L. A., Thingstad F. (1983) The ecological role of the water-column microbes in the sea. Mar. Ecol. Prog. Ser. **10:** 257–263

Butler H., Rogerson A. (1995) Temporal and spatial abundance of naked amoebae (Gymnamoebae) in marine benthic sediments of the Clyde Sea area, Scotland. J. Eukaryot. Microbiol. **42**: 724-730

Fenchel T. (1987) Ecology of Protozoa: The Biology of Free-living Phagotrophic Protists. Science Tech Publishers/Springer Verlag, Madison/Wisconsin, Berlin

Gage J. D., Tyler P. A. (1991) Deep-Sea Biology: A Natural History of Organisms at the Deep-Sea Floor. Cambridge, UK: Cambridge University Press

Guindon S., Gascuel. O. (2003) A simple, fast and accurate algorithm to estimate large phylogenies by maximum likelihood. Systems Biology, **52**: 696-704.

Hausmann K., Hülsmann N., Polianski, I., Schade S., Weitere M. (2002) Composition of benthic protozoan communities along a depth transect in the eastern Mediterranean Sea. Deep Sea Research I **49**: 1959-1970

Hausmann K., Selchow P., Scheckenbach F., Weitere M., Arndt H. (2006) Cryptic species in a morphospecies complex of heterotrophic flagellates: The case study of *Caecitellus spp*. Acta Protozool. **45**: 415-431

James-Clark, H. (1867). On the affinities of the sponges. On the Spongiæ Ciliatæ as Infusoria Flagellata. Mem. Boston Soc. Nat. Hist. **I**: 305-340.

Kent W.S. 1880 (1880-82) Manual of the Infusoria. Vol 1. Bogue, London

Kumar S., Tamura K., Nei N. (2004) MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. Briefings in Bioinformatics **5**: 150-163

Leadbeater B. S. C. (1990) Choanoflagellate organization with special reference to loricate taxa. In: The Biology of Free-living Heterotrophic Flagellates, (Eds. D. J. Patterson, J. Larsen) Clarendon Press Oxford, 241-258

López-García P., Rodriguez-Valera F., Pedros-Alio C., Moreira D. (2001) Unexpected diversity of small eukaryotes in deep-sea Antarctic plankton. Nature **409:** 603-607

Massana R., Terrado R., Vovejoy C., Pedros-Alio C. (2006) Distribution and abundance of uncultured heterotrophic flagellates in the world oceans. Environ. Microbiol. **8:** 1515-1522

Nylander J. A. A. (2004) MrAIC.pl. Program distributed by the author. Evolutionary Biology Centre, Uppsala University, Uppsala

Patterson D. J., Nygaard K., Steinberg G., Turley C. M. (1993) Heterotrophic flagellates and other protists associated with oceanic detritus throughout the water column in the mid North Atlantic. J. Mar. Biol. Ass UK **73:** 67-95

Scheckenbach F., Wylezich C., Weitere M., Hausmann K., Arndt H. (2005) Molecular identity of strains of heterotrophic flagellates isolated from surface waters and deep-sea sediments of the South Atlantic based on SSU rDNA. Aquat. Microb. Ecol. **38:** 239-247

Scheckenbach F., Wylezich C., Mylnikov A. P., Weitere M., Arndt H. (2006) Molecular comparisons of freshwater and marine isolates of the same morphospecies of heterotrophic flagellates. Appl. Environ. Microbiol. **72**: 6638-6643

Skuja H. (1956) Taxonomische und Biologische Studien über das Phytoplankton Schwedischer Binnengewässer. Nova Acta Reg. Soc. Sci. Upsal. **IV 16**: 1-404

Smith C. R., Demopoulos A. (2003) Ecology of the deep Pacific Ocean floor. In: Ecosystems of the World, Volume 28: Ecosystems of the Deep Ocean, ed. PA Tyler, Amsterdam, the Netherlands: Elsevier, 179-218

Stoeck T., Fowle W. H., Epstein S. S. (2003) Methodology of protistant discovery: from rRNA detection to quality scanning electron microscope images. Appl. Environ. Microbiol. **96:** 6856-6863

Thompson J. D., Gibson T. J., Plewniak F., Jeanmougin F. ,Higgins D. G. (1997) The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Research **24**: 4876-4882

KAPITEL IV

Bipolar comparisons of acanthoecid choanoflagellates

ABSTRACT

Bipolar distribution gives the unique opportunity to study allopatric speciation processes. We chose the choanoflagellate morphospecies of *Acanthocorbis nana* and *A. unguiculata* to investigate, whether an independent evolution of bipolar morphospecies after the establishment of the cold-water provinces in late Miocene took place. The genetic variance in the small subunit ribosomal RNA gene was used to identify genotypes. Genotypes of *Acanthocorbis nana* and *A. unguiculata*, from Arctic and Antarctic sampling sites differed significantly from each other. It seems that the tropic formed a physical barrier for the gene flow of these bipolar species. On the other hand, only one genotype was found for the worldwide distributed choanoflagellate species, *Diaphanoeca grandis*, indicating a continuous gene flow between polar populations.

Keywords Bipolarity, choanoflagellates, morphology, biogeography, Antarctica, Arctic, Acanthoecidae, cryptic species

INTRODUCTION

The formation of the polar regions with its separation of cold water masses may be considered as a large scale experiment of speciation. However, there are only a few groups of organisms which find comparative environmental conditions for their live at both poles. Marine choanoflagellates are such a group of organisms feeding on small bacteria in polar waters of both poles. Choanoflagellates may serve as target organism to search for the effect of longterm separation. On the other hand, choanoflagellates belong to a size class of organisms assumed to show a ubiquitous dispersal pattern according to their environmental needs. According to the assumption that the high reproduction rate and abundance combined with a high dispersal rate should lead to a ubiquitous distribution pattern, both polar regions with their comparable environmental conditions should show a similar species composition.

Choanoflagellates form an important and ubiquitous group of heterotrophic flagellates (for review see Leadbeater (16)). They compose between about 5 and 40 per cent of nanoflagellate biomass in marine, brackish and freshwater pelagic waters (1) and are an abundant component of the Antarctic marine plankton (13, 18). Choanoflagellates consume the smallest size fraction of bacteria as typical filter-feeders (3, 8, 10) and act as important nutrient remineralisers and intermediaries of bacterial production to higher trophic levels (2, 10).

There are only about 200 valid species of choanoflagellates, of which 103 species in 29 genera belong to acanthoecid choanoflagellates. Acanthoecids have mainly been described on the basis of their basket-like lorica composed of siliceous ribs or costae (cf. 16). The remaining species belong to codonosigid and salpingoecid choanoflagellates found not only in marine but also in fresh and brackish waters. Recent molecular biological studies have indicated that morphospecies of heterotrophic nanoflagellates are often represented by very different genotypes with sometimes significant ecological differences (14, 19, 24, 25).

Bipolar distribution of organisms in marine habitats is found across marine planktonic protists as well as in invertebrate, vertebrate, and plant groups (17). It is uncertain whether these species are isolated within their water masses or whether an exchange across the tropics occurs. The morphological characteristics of bipolar species may be the result of a convergent or parallel evolution due to similar environmental factors. If the morphospecies were isolated in their cold water provinces and no gene flow took place one should expect a certain genetical divergence in the two separated populations. On the other hand, when considering a gene flow across the tropics the populations should be genetically homogenous. Studies on planktonic

foraminifers indicated a gene flow between the polar regions across the tropics for cosmopolitan species (6), while within once connected and now separated populations of bipolar species of foraminifers a speciation took place resulting in the evolution of cryptic species (7, 22). Other studies on heterotrophic nanoflagellates (4) showed,that protistan transport to Antarctica may be restricted and thus allow local protistan populations to adapt to local environmental conditions and to evolve cryptic species.

Previous studies on the biogeographic distribution of acanthoecid choanoflagellates (21) provided the information that *Acanthocorbis unguiculata* and *A. nana* were recorded only from both coldwater regions, while *Diaphanoeca grandis* showed a ubiquitous distribution. SSU rRNA was used to characterize genotypes

MATERIAL AND METHODS

Sampling sites. Samples from Sub- and Antarctic waters were taken by Mark Felix from Weddell Sea on R/V Polarstern (cruise ANT XXI) in Austral Summer 2003/2004. Surface water samples were collected at the following positions: site I (70° 49'S/18° 43'W) and site II (54° 44'S/0° 7'E). Artic samples were taken by Eva Leu (School of Fishery, Tromsø, Norway) in July 2005 at the following position: site IE (82°05'N/11°36'E), where different depths were sampled (0m, 200m 500m). Samples were aliquoted and species were isolated and transferred to cell culture flasks (50 ml, Sarstedt). A sterilized wheat grain was provided as nutrition for autochthonous bacteria populations as a food source for choanoflagellates. Each week, samples were examined by light microscopy (Zeiss Axiovert 200). Samples containing choanoflagellates were registered and diluted with artificial seawater of the same salinity. Three cultures of *Acanthocorbis nana* and three cultures of *A. unguiculata* were established. In addition, two cultures of *Diaphanoeca grandis* were established. Long-term storage of samples and cultures was carried out at 4°C in a 12/12 hour day-night cycle.

Electron microscopy. The preparation of cells basically followed the protocol used by Stoeck et al. (26). Samples were fixed with 50:50 Bouin's fixative (buffered formaldehyde saturated with picric acid and 2% glacial acetic acid added immediately before fixation) with the addition of 0.1 to 0.2% glutaraldehyde, for 45 minutes at 4°C. Samples remained in the culture flask and a dehydration series of ethanol with 30%, 50%, 60%, 70%, 80%, 90%, 96% and pure ethanol (each step was repeated two times and lasted 10 minutes) was carried out. After this procedure, a 50:50 hexamethyldisilazane (HMDS)-Ethanol solution was applied for 30 minutes followed by

pure HMDS for 30 minutes. Afterwards, the samples were allowed to dry. The bottom of the flasks was cut to appropriate size and stuck to a sample holder. SEM samples were sputtered with a 120Å layer of gold before examination by SEM (Hitachi S-520).

Molecular biology. Between 3 to 21 specimens were analyzed by means of **s**ingle cell PCR. Organisms were extracted from each culture using a micromanipulator. Each single cell was transferred to 27µl sterilized water and frozen at -20°C for three hours before PCR. The SSU rDNA fragment was amplified using 18SFor (AACCTGGTTGATCCTGCCAGT) and 18SRev-Ch (CCGTAGGTGAACCTGCAGAAGGA) primer at a concentration of 1.6 nM followed by a reamplification with the primer pair 82F (GAAACTGCGAATGGCTC) and 18Srev-1 (CGTAACAAGGTTT CCGTAGGT). The PCR products were purified using the E.Z.N.A. Cycle-Pure-Kit (Peqlab, Erlangen, Germany). The sequencing of rDNA was done using Big Dye-Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Weiterstadt, Germany) in accordance with the manufacturer's instructions. For sequencing the Primer 590F (CGGTAATTCCAGCTCCAATAGC) was used. The obtained sequences were manually arranged.

Phylogenetic analyses. Alignments were carried out using ClustalX (27) and corrections were made manually with BioEdit. The model (JC69) for maximum likelihood analysis was determined by MrAIC (23) and the ML analysis computed by PhyML (12), using 100 replicates for the bootstrap analysis. Minimum evolution (NJ) was calculated using MEGA 3.1 (15) using the K2P model and 1000 replicates for bootstrap analysis.

RESULTS

Electron microscopy identified the clones HFCC412, HFCC417 and HFCC401 as *Acanthocorbis nana* (Fig.1 A and C). The main criteria for morphological identification were the rounded tips of the anterior longitudinal costae and the diagonal arrangement of the anterior latitudinal costae of the lorica. The other three clones, HFCC418, HFCC413 and HFCC411, were identified as *Acanthocorbis unguiculata* (Fig.1 D and F). The identification criteria for this species were the pointed tips of the anterior longitudinal costae and the horizontal arrangement of the anterior latitudinal costae of the lorica (Leadbeater pers. comm.). The cultures HFCC400 and HFCC422 were assigned to the species *Diaphanoeca grandis* on the basis of electron microscopy (Fig.1 G and H).

Thirty-nine specimens of *Acanthocorbis nana* and thirty-five specimens of *A. unguiculata* from Atlantic Arctic and Antarctic waters were examined. The partial 18S rDNA phylogenetic bootstrap analysis revealed 3 SSU genotypes among the

morphospecies *Acanthocorbis nana* (Fig. 2) The strain from Subantarctic surface waters (HFCC401) was in the same cluster as one strain from the Arctic originating from a depth of 200m (HFCC417) but there was p-distance of 2.1 percent between these two strains. The p-distance of both other sequences to the third clone from Arctic surface waters (HFCC412) was 3.6 percent (Tab. 1). *Acanthocorbis nana* clustered together with sequences from species identified as *Acanthoeca spectabilis* (Fig. 2).

Within the three cultures of *A. unguiculata* two different SSU genotypes were found (Fig. 2). The strain from Arctic waters from 200m (HFCC418) clustered together with the Arctic strain originating from a depth of 500m (HFCC413) and was nearly identical. The p-distance of the Arctic genotypes to the clone from Antarctic surface water (HFCC411) was 6 percent (Tab. 2).

Table 1. p-distance between the genotypes of *Acanthocorbis nana*

	North Atlantic NCBI	Subantarctic 0m (n=12)	Arctic 0m (n=21)	Arctic 200m (n=6)
	L10823	**HFCC401**	**HFCC412**	**HFCC417**
L10823	0			
HFCC401	0.036	0		
HFCC412	0	0.036	0	
HFCC417	0.036	0.021	0.036	0

Table 2. p-distance between the genotypes of *Acanthocorbis unguiculata*

	Antarctic 0m (n=15)	Arctic 200m (n=3)	Arctic 500m (n=17)
	HFCC411	**HFCC418**	**HFCC413**
HFCC411	0		
HFCC418	0.062	0	
HFCC413	0.060	0.002	0

DISCUSSION

We found that there are three genotypes of *Acanthocorbis nana*. There is a difference in genotype between the Antarctic and Arctic samples but there was also a difference within the Arctic samples which only differed in the vertical position (surface and 200m depth). In the morphotype of *A. unguiculata* we found two genotypes, divided between the polar regions. *Diaphanoeca grandis* did no show a significant variation in the SSU rRNA.

Considering the genetic distances between the genotypes of *Acanthocorbis unguiculata* and between the genotypes *of A. nana* from the different coldwater provinces it appears to be likely that the genetic separation took place before the last formation of the coldwater provinces in late Miocene. We used an estimated value for the nucleotide substitutions of SSU rRNA to testify the hypothesis of the temporal separation. As the available evolutionary rates reflect the evolution of the whole SSU rRNA (9) and our analysis was based on partial SSU rRNA, we calculated the divergence times using three alternative evolutionary rates, 0.2%, 1% and 1.8% sequence divergence per 100 Ma. Exact rates can not be given due to missing fossil records from choanoflagellates which would be the base for a molecular clock calibration. The only records of possible fossil choanoflagellates (11) are based on the study of a cyst which might originate from choanoflagellates (only about 5-3Ma old), but this finding could not be verified. For an estimate of the divergence time within the two *Acanthocorbis* species, we used the equation r=K/2T, where r = evolutionary rate of SSU rRNA, K = corrected distances and T = divergence time. Since the highly variable regions of SSU rRNA were excluded from the alignment a conservative estimate of the evolutionary rate of 0.2 % sequence divergence per 100 Ma (linearised tree) was assumed. For the calculations the function of molecular clock implemented in MEGA 3.1 (15) was used. The estimate of the divergence time for *A. nana* dated the separation of the Antarctic strain (HFCC401) and the Arctic strain (HFCC417) at about 10.4Ma. For the strains of *A. unguiculata* from Arctic (HFCC418, 413) and Antarctic (HFCC411) waters, a divergence time of about 16.9 Ma was calculated (Fig. 3). These estimates made it likely that the beginning of speciation of the two *Acanthocorbis* species dated back to the time of the last formation of the coldwater provinces in late Miocene (16-8Ma).

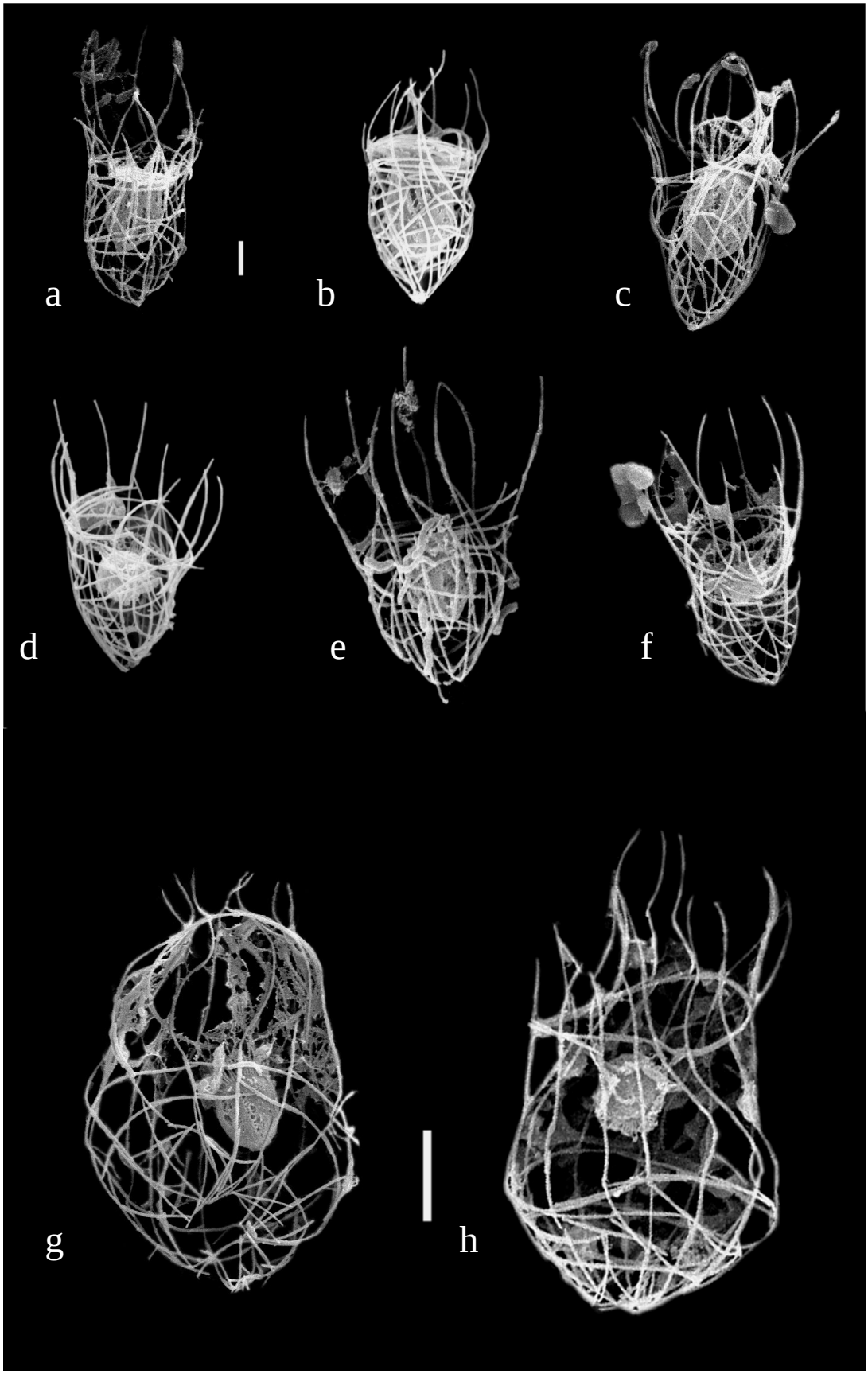

Figure 1. a-c: Scanning electron micrographs of *Acanthocorbis unguiculata* a: HFCC418; b: HFCC411; c: HFCC413; d-f: Scanning electron micrographs of. *Acanthocorbis nana*. d: HFCC412; e: HFCC417; f: HFCC401; Scalebar 2µm. g-h: Scanning electron micrographs of.*Diaphanoeca grandis* g: HFCC400; h: HFCC103; Scalebar 5µm

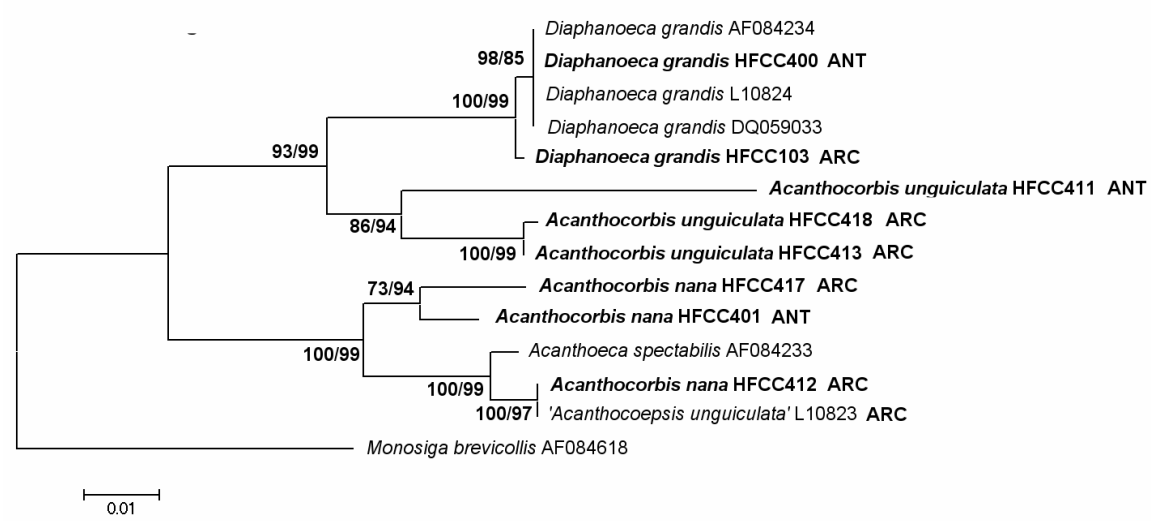

Figure 2. Phylogenetic tree of partial 18S rRNA (~600bp) numbers indicate ML/NJ values (see material and methods). ANT and ARC indicate the origin from the southern and northern hemisphere, respectively. Scale bar indicates the genetic distance.

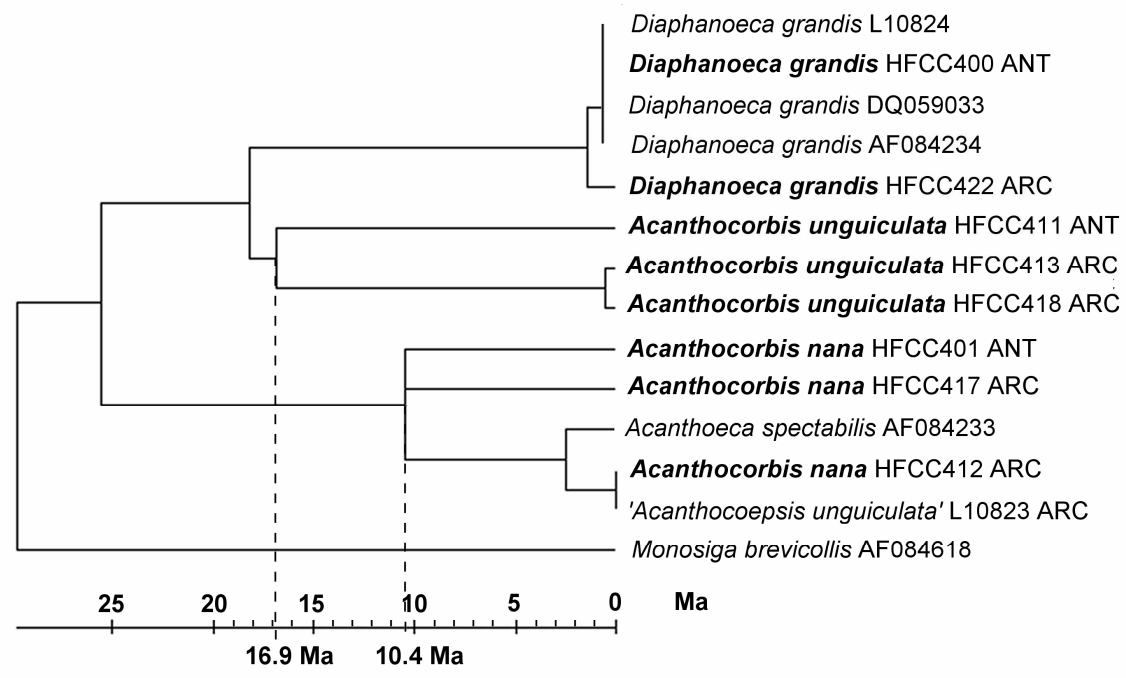

Figure 3. Linearized NJ tree of partial 18S rRNA (~600bp) for molecular dating. Scale bar in Million Years (MA).

When we consider the present oceanographic situation (cf. 5), there is a surface water exchange between the two cold water provinces. The cold surface water from Antarctic and Arctic waters meet the warm tropic currents, making it likely that coldwater adapted species as *A. unguiculata* and *A. nana* do not pass this physical barrier in a high number. The other way of water exchange between the polar regions are the deep sea flows connecting both regions. The absence of acanthoecid choanoflagellates from deep sea regions (until now no acanthoecid species was found in deep sea samples) makes it likely that main way of distribution for the two species from the genus *Acanthocorbis* are the surface currents and therefore limiting the gene exchange between the polar regions. To testify this hypothesis we will try to study the ecology of *A. nana* and *A. unguiculata* regarding salinity resistance, temperature tolerance and possible cyst formation.

The finding of different genotypes of the morphospecies *Acanthocorbis nana* and *A. unguiculata* from the separated polar regions suggest, that a diversification in Arctic and Antarctic waters took place. The number of species and hence the amount of biogeographic restricted species might be underestimated. Regarding the variance in genotype of acanthoecid choanoflagellates within polar regions the theory on "ubiquitous" dispersal for most free–living marine protists should be reconsidered.
The presents of "*Acanthocoepsis unguiculata"* (synonym from *Acanthocorbis. unguiculata)* in the same cluster as our *A. nana* strains was due to a misidentification, as it had been re-identified as *A. nana* (pers. comm. Leadbeater).

Studies on bipolar foraminifers and dinoflagellates showed that some species are able to pass the physical barrier and thus continuously exchange their genes (6, 20). But there are also foraminifers and heterotrophic nanoflagellates, which are restricted to their cold water habitats and are unable to pass the tropics (4, 7, 22). Our study gives evidence, that there is a similar speciation within acanthoecid choanoflagellates. Some coldwater species like *Acanthocorbis nana* and *A. unguiculata* are likely to show only a very limited gene exchange through the tropics, allowing a speciation between the cold water regions and thus forming cryptic species. Other species with a world wide distribution like *Diaphanoeca grandis* do not vary within the genotype making it likely that even the polar specimens share the same gene pool.

ACKNOWLEDGEMENTS

We thank Barry S.C. Leadbeater for valuable discussions and constructive comments. We also thank the German Research Foundation (DFG) for supporting this project with a grant to H.A (AR 288/8 and AR 288/12).

REFERENCES

1. **Arndt, H., D. Dietrich, B. Auer, E. Cleven, T. Gräfenhan, M. Weitere and A. P. Mylnikov.** 2000. Functional diversity of hetrotrophic flagellates in aquatic ecosystems, p. 240-268. *In* B. S. C.,Leadbeater and. J. C. Gree (ed.) The Flagellates. Taylor & Francis Ltd, London,.
2. **Azam, F., T. Fenchel, J. G. Field J. S. Gray, L. A .Meyer-Reil and F. Thingstad.** 1983. The ecological role of the water-column microbes in the sea. Mar. Ecol. Prog. Ser. **10**: 257–263.
3. **Boenigk, J. and H. Arndt.** 2000. Comparative studies on the feeding behaviour of two heterotrophic nanoflagellates: the filterfeeding choanoflagellate *Monosiga ovata* and the raptorial-feeding kinetoplastid *Rhynchomonas nasuta*. Appl. Environ. Microbiol. **22**: 243–249.
4. **Boenigk, J., K. Pfandl,T. Garstecki, H. Harms, G. Novarino and A. Chatzinotas**. 2006. Evidence for Geographic Isolation and Signs of Endemism within a Protistan Morphospecies. Appl. Environ. Microbiol. **72**: 5159–5164
5. **Broecker, W. S**. 1991. The great ocean conveyor. Oceanogr. **4**: 79-88.
6. **Darling, K. F., C. M. Wade, I. A. Stewart, D. Kroon, R. Dinglek and A. J. Leigh Brown.** 2000. Molecular evidence for genetic mixing of Arctic and Antarctic subpolar populations of planktonic foraminifers. Nature **204**: 43-47.
7. **Darling, K. F., M. Kucera, C. J. Pudsey and C. M. Wade**. 2004. Molecular evidence links cryptic diversification in polar planktonic protists to Quaternary climate dynamics. Proc. Natl. Acad. Sci. **101**: 7657–7662.
8. **Ecclestone-Parry, J. D. and B. S. C. Leadbeater.** 1994. A comparison of the growth kinetics of six marine heterotrophic nanoflagellates fed with one bacterial strain. Mar. Ecol. Prog. Ser. **105**: 167-177

9. **Escalante, A. A. and F. J. Ayala**. 1995. Evolutionary Origin of Plasmodium and Other Apicomplexa Based on rRNA Genes. Proc. Natl. Acad. Sci. **92**: 5793-5797.
10. **Fenchel, T.** 1987. Ecology of Protozoa: The Biology of Free-living Phagotrophic Protists. Science Tech Publishers/Springer Verlag, Madison/Wisconsin, Berlin
11. **Fenner, J.** 1991. 15. Rare and unknown noncalcareous microfossiles recovered from leg 114 sites. Proceedings of the Ocean Drilling Program, Scientific Results **114**: 303-310.
12. **Guindon, S. and O. Gascuel.** 2003. A simple, fast and accurate algorithm to estimate large phylogenies by Maximum Likelihood. Syst. Biol. **52**: 696-704.
13. **Hewes, C. D., E. Sakshaug, F. M. H. Reid and O. Holm-Hansen.** 1990. Microbial autotrophic and heterotrophic eucaryotes in Antarctic waters: relationships between biomass and chlorophyll, adenosine triphosphate and particulate organic carbon. Mar. Ecol. Prog. Ser. **63**: 27-35.
14. **Hausmann, K., P. Selchow, F. Scheckenbach, M. Weitere and H. Arndt.** 2006. Cryptic specis in a Morphospecies Complex of Heterotrophic Flagellates: The case study of *Caecitellus* spp. Acta Protozool.
15. **Kumar, S., K. Tamura and N. Nei.** 2004. MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. Briefings in Bioinform. **5**: 150-163
16. **Leadbeater, B. S. C.** 1991. Choanoflagellate organization with special reference to loricate taxa, p. 241–258. *In* D. J. Patterson and J. Larsen (ed.), Freeliving heterotrophic flagellates. Clarendon Press, Oxford.
17. **Lindberg, D. R.** 1991. Marine biotic interchange between the northern and southern hemispheres. Paleobiol. **17**: 308-324.
18. **Marchant, H. J.** 1985. Choanoflagellates in the Antarctic marine food chain, p, 217-276. *In* W. R. Siegfried, P. R. Condy and R. M. Laws (ed.), Antarctic nutrient cycles and food webs. Springer-Verlag, Berlin.
19. **Massana, R., R. Terrado, C. Vovejoy and C. Pedros-Alio.** 2006. Distribution and abundance of uncultured heterotrophic flagellates in the world oceans. Environ. Microbiol. **8:** 1515-1522
20. **Montresor, M., Lovejoy, C., Orsini, L., Procaccini, G. and S. Roy.** 2003. Bipolar distribution of the cyst-forming dinoflagellate *Polarella glacialis*. *Pol. Biol*. **26**: 186–194.

21. **Nitsche, F. and H. Arndt.** subm. Biogeographical distribution of Acanthoecidae (Choanoflagellida) morphospecies with special interest in Polar Regions.
22. **Norris, R. D. and C. de Vargas**. 2000. Evolution all at sea. Nature **405**: 23-24.
23. **Nylander, J. A. A.** 2004. MrAIC.pl. Program distributed by the author. Evolutionary Biology Centre, Uppsala University, Uppsala.
24. **Scheckenbach, F., C. Wylezich, M. Weitere, K. Hausmann and H. Arndt.** 2005. Molecular identity of strains of heterotrophic flagellates isolated from surface waters and deep-sea sediments of the South Atlantic based on SSU rDNA. Aquat. Microb. Ecol. **38**: 239-247.
25. **Scheckenbach F, C. Wylezich, A. P. Mylnikov, M. Weitere and H. Arndt.** 2006. Molecular comparisons of freshwater and marine isolates of the same morphospecies of heterotrophic flagellates. Appl. Environ. Microbiol. **72**: 6638-6643.
26. **Stoeck, T., W. H. Fowle and S. S. Epstein.** 2003. Methodology of protistant discovery: from rRNA detection to quality scanning electron microscope images. Appl Env Microb **96**: 6856-6863.
27. **Thompson, J. D., T. J. Gibson, F. Plewniak , F. Jeanmougin and D. G. Higgins.** 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Research **24**: 4876-4882

KAPITEL V

Studies on the phylogenetic relationships among choanoflagellates

Abstract

It is broadly accepted that multicellular life derived from choanoflagellate like protists that were the ancestors of simple choanocyte-bearing metazoans. Ultrastructural and molecular biological studies of the rRNA and other genes confirmed this hypothesis. Yet the exact phylogenetic position of choanoflagellates is uncertain. Whether they are based at the root of metazoans or form a sister-group could not be resolved. The monophyletic origin of metazoans seems to be confirmed by many molecular biological studies. In our study we used a high number of SSU and LSU rRNA sequences from choanoflagellates first to resolve the phylogeny within choanoflagellates and second to analyse their position within the clade of Opisthokonta which is containing Metazoa, fungi, Microsporidia, choanoflagellates and Choanozoa. We found, that the classical families of choanoflagellates, Salpingoecida and Codonosigida, are not coherent on the molecular biological level. The taxonomy of these two families is in urgent need of revision. The classical morphological characteristics defining members of these families, mainly the investment, are not sufficient to describe these taxonomic units. Our analysis based on the SSU rRNA of choanoflagellate sequences together with other opisthokont and metazoan sequences makes it likely that Choanozoa together with Fungi form a sister group to the Metazoa.

Introduction

In the late 19th century the remarkable similarity in cell architecture between choanoflagellates and choanocytes of sponges was observed for the first time (Clark 1866). This led to the hypothesis that sponges, the simplest metazoans, evolved from choanoflagellate-like protist ancestors (Clark 1868; Haeckel 1874). On the other hand this similarity was also used to support the opposite hypothesis, that sponges are colonial choanoflagellates (Kent 1878). Later on, the association of sponges to the order of metazoans was founded on the basis of spermatogenesis and the similarity between choanocytes and choanoflagellates was considered to be an analogy (Schulze 1885). Only in the second half of the 20th century structural and functional studies showed that this similarity is a homology (Nielsen 1987). This led to the hypothesis that choanoflagellates are ancestors of sponges and all other metazoans. Current ultrastructural and molecular biological studies make it most likely that metazoans have a monophyletic origin (Salvini-Plawen 1978; Ax 1989; Wainright *et al*. 1993; Kumar & Rzhetsky 1996; Ragan *et al*. 1996; Medina *et al*. 2001; Lang *et al*. 2002). Molecular phylogenies support a common ancestry between animals (Metazoa) and fungi (Baldauf et al. 1993; 2000), but the evolutionary descent of the Metazoa from single-celled eukaryotes (protists) and the nature and taxonomic affiliation of these ancestral protists remain uncertain. There are three different hypotheses for the relationship between choanoflagellates, fungi and animals (Fig. 1) which were used as a basis for the present study (Maldonado 2004).

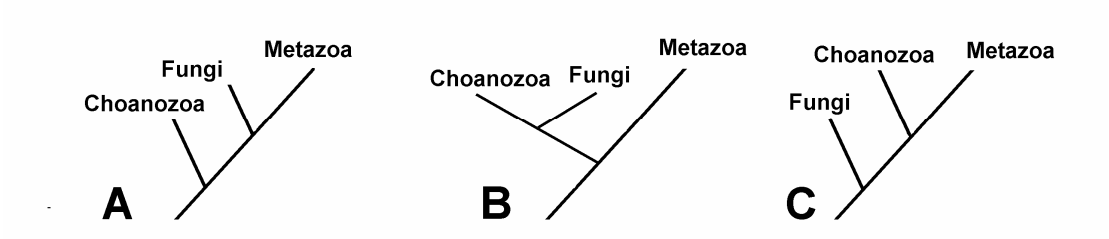

Figure 1. Three different possible scenarios for the relationship within Opisthokonta. A: Choanozoa form the base from wich Fungi and then Metazoa evolved. B: Choanozoa and Fungi form a own branch as sistergroups to the Metazoa. C: Fungi form the base from wich Choanozoa and then Metazoa evolved (mod. after Maldonado 2004).

In the course of our study of marine and freshwater choanoflagellates the data set of genotypes available for a comparative study of choanoflagellates was significantly enlarged. This offered the unique chance to study the comparative phylogeny of choanoflagellates.

At present there are 5 classes within the phylum of Choanozoa which are Aphelidea, Corallochytrea, Mesomycetocoa, Cristidiscoidea and Choanoflagellatea (Cavalier-Smith & Chao 2003). The class Mesomycetocoa containing Dermocystidium, Ichthyophonus, and Psorospermium, all protistan parasites of fish and crustaceans, has been placed somewhere near the divergence of animals and fungi (Ragan *et al*. 1996). The Amoebidiales had traditionally been classified as trichomycete fungi but are now placed in the phylum Choanozoa. In previous studies the relationship among Choanoflagellata, Mesomycetocoa and animals was critically addressed using complete SSU and large subunit (LSU) rRNA data, either individually or in combination (Medina *et al*. 2001). Their study demonstrated the pitfalls of tree evaluations which are based on bootstrap and nonparametric bootstrap values and the difficulties if not impossibility of the precise interpretation of such values (Whelan 2001). Different test models may lead to conflicting tree topology and thus showing that the available data may be insufficient to resolve the question of relationships between these classes.

The class of Choanoflagellatea consists currently of three families characterized by their morphology. The Codonosigidae or naked choanoflagellates are described to have a thin, electron transparent investment. The Salpingoecidae have a so called theca, an organic housing. Both families are distributed worldwide in fresh water, marine and brackish waters. The third and last family are the Acanthoecidae, characterized by the presence of a siliceous lorica. They have been found only in marine and brackish waters.

In our study we used partial SSU rRNA to add some knowledge to resolve the systematic position of choanoflagellate families and to contribute to an understanding of the origin of the metazoan tree of life.

Methods

Water samples from many regions all over the world were searched for choanoflagellates (Fig.1). The protists were isolated to established cultures and were either extracted directly from the sample or from the cultures using a micromanipulator. Single cells were transferred to 27µl sterilized water and frozen at -20°C for three hours before PCR. The SSU rDNA fragment was amplified using 18SFor (AACCTGGTTG ATCCTGCCAGT) and 18SRev-Ch (CCGTAGGTGAACCTGCAGAAGGA) as primers at a concentration of 1.6 nM followed by a reamplification with the primer pair 82F (GAAACTGCGAATGGCTC) and 18Srev-1 (CGTAACAAGGTTTCCGTAGGT).

Additionally, the high variable region D3 to D5 of LSU rDNA was amplified using D3 (GGCTACCATC CGTAGGACTATGA) and D5 (CTTAAGCATATCAATAAGCGG) primer also at a concentration of 1.6 nM. The PCR products were purified using the E.Z.N.A. Cycle-Pure-Kit (Peqlab, Erlangen, Germany). The sequencing of rDNA was done using Big Dye-Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Weiterstadt, Germany) in accordance with the manufacturer's instructions. Primers used for sequencing were 590F (CGGTAATTCCAGCTCCAATAGC) and D5. The obtained sequences were manually arranged.

Phylogenetic analyses. Alignments were carried out using ClustalX (Thompson *et al.* 1997) and corrections were made manually with BioEdit. For the phylogenetic position of Choanozoa within Opisthokonta, the model (GTRIG) for maximum likelihood analysis was determined by MrAIC (Nylander 2004) and the ML analysis computed by PhyML (Guindon and Gascuel 2003) using 100 replicates for the bootstrap analysis. Minimum evolution (NJ) was calculated using MEGA 3.1 (Kumar *et al.* 2004) using the LogDet model and 1000 replicates for bootstrap analysis. For the intraspecific analysis the JC69 (ML) and K2P (NJ) models were applied.

Results

In addition to the available sequences from gene banks, we sequenced the partial SSU rRNA of seventeen choanoflagellate species, of which three are newly discovered species (Tab. 1). In addition, seven sequences of the partial LSU were provided (Tab. 1). Based on this dataset our analysis showed that the taxonomy within the class of Choanoflagellatea is in a strong need of a revision. The members of the families of salpingoecid and codonosigid choanoflagellates did not cluster together. There might be a separation of freshwater and marine species in these two families, but due to the lack of ecological data no final conclusions could be drawn (Fig. 3).

The acanthoecid choanoflagellate species morphologically characterized by their siliceous lorica formed a distinct cluster. Only the two species of the genus *Diplotheca* did not cluster within the family of acanthoecids. Their very specific lorica composition and structure may indicate a separate lineage of evolution. The intraspecific bootstrap analysis using partial LSU rRNA confirmed the uncertain status of the families of salpingoecid and codonosigid choanoflagellates. And it confirmed the acanthoecid choanoflagellates as a valuable taxonomic unit (Fig. 4). The bootstrap analysis of the SSU rRNA supported the hypothesis of a monophyletic origin of Metazoa. Choanozoa and Fungi clustered on an own branch in the tree distinctly separated from the Metazoa. Within the phylum of Choanozoa, all classes (Aphelidea, Corallochytrea,

Mesomycetocoa, Cristidiscoidea and Choanoflagellatea) cluster together underlining the current ideas of opisthokont systematics. This is supported by the high bootstrap values (Fig. 2).

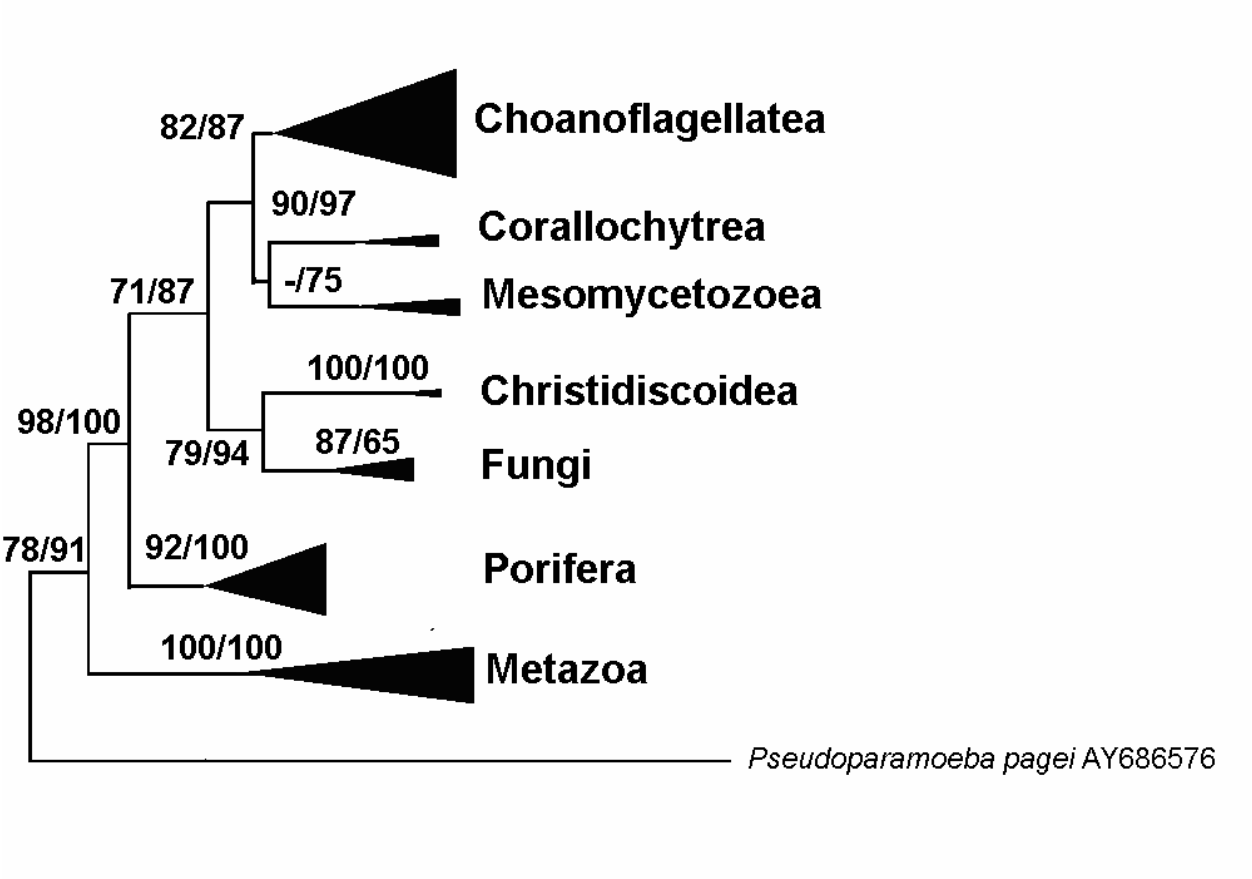

Figure 2. Phylogenetic bootstrap analysis of partial SSU rRNA regarding the position of Choanozoa in the group of Opisthokonta using sequences listed in Table 1 and 2 and in Figure 3. First value for NJ, second for ML (see methods). Missing numbers indicate values under 50%.

Table 1. List of own choanoflagellate species used for SSU and LSU rRNA analysis with sampling site and strain number (HFCC=Heterotrophic Flagellate Culture Cologne)

Species	**Sampling site**	**SSU**	**LSU**	**Strain**
Acanthocorbis unguiculata	Weddell Sea	x		HFCC411
Acanthocorbis unguiculata	Arctic Atlantic	x		HFCC413
Acanthocorbis unguiculata	Arctic Atlantic	x		HFCC418
Acanthocorbis nana	Weddell Sea	x	x	HFCC401
Acanthocorbis nana	Arctic Atlantic	x		HFCC412
Acanthocorbis nana	Arctic Atlantic	x		HFCC417
Codonosiga botrytis	River Rhine at Cologne	x	x	HFCC43
Diaphanoeca grandis	Weddell Sea	x	x	HFCC400
Diaphanoeca grandis	North Atlantic	x	x	HFCC103
Diaphanoeca grandis	Arctic Atlantic	x	x	HFCC422
Diaphanoeca pedicellata	Arctic Antlantic	x	x	HFCC409
Diplotheca costata	Pesian Gulf	x		HFCC431
Diplotheca elongata sp. nov	Estuary of River Danshui, Taiwan	x		HFCC430
Lagenoeca antarctica sp. nov	Weddell Sea	x		HFCC429
Monosiga ovata	River Rhine	x	x	HFCC41
Monosiga sp. nov.	River Rhine at Cologne	x	x	HFCC45
Salpingoeca abyssalis sp. nov.	South Atlantic	x		HFCC426
Salpingoeca marina	Mediterranean Sea	x		HFCC406
Salpingoeca pyxidium	-ATCC- culture-	x		HFCC446
Salpingoeca urceolata	-ATCC- culture-	x		HFCC445
Salpingoeca sp.	River Wye, Wales	x		HFCC415
Salpingoeca sp.	Weddell Sea	x		HFCC441
Stephanoeca cf. *pyxidoides*	Mediterranean Sea	x	x	HFCC437

Table 2. List of species used for SSU rRNA analysis with accession number and systematic position

Species	Accession number	Systematic
Anemonia sulcata	X53498	Cnidaria
Caenorhabditis elegans	AY284652	Nematoda
Chaetopterus pugaporcinus	DQ209223	Annelida
Rhyssoplax olivacea	DQ779644	Mollusca
Sepia officinalis	AY557471	Mollusca
Gibbula cineraria	AY340430	Mollusca
Zonophryxus quinquedens	DQ008451	Arthropoda
Ilyarachna antarctica	AY461481	Arthropoda
Calopteryx aequabilis	AJ458978	Arthropoda
Albula vulpes	X98842	Vertebrata
Homo sapiens	HSRRN18S	Vertebrata
Mus musculus	AY248756	Vertebrata
Ute ampullacea	AM180972	Calcarea
Grantiopsis sp.	AM180977	Calcarea
Sycettusa tenuis	AM180975	Calcarea
Xestospongia muta	AY621510	Demospongiae
Verongula gigantea	AY591804	Demospongiae
Trochospongilla pennsylvanica	DQ087507	Demospongiae
Trochospongilla horrida	AY609320	Demospongiae
Tethya actinia	AY878079	Demospongiae
Smenospongia aurea	AY591806	Demospongiae
Geodia neptuni	AY878078	Demospongiae
Ephydatia fluviatilis	AY578146	Demospongiae
Ephydatia cooperensis	AF140354	Demospongiae
Aplysina lacunosa	AY591803	Demospongiae
Aplysina cavernicola	AY591800	Demospongiae
Aiolochroia crassa	AY591805	Demospongiae
Ministeria vibrans	AF271998	Christidiscoidea
Dermocystidium percae	AF533949	Mesomycetocoa
Paramoebidium sp.	AY336708	Mesomycetocoa
Amphibiothecum penneri	AY772001	Mesomycetocoa
Dermocystidium sp.	AF533950	Mesomycetocoa
Pseudoperkinsus tapetis	AF192386	Mesomycetocoa
Ichthyophonus irregularis	AF232303	Mesomycetocoa
Anurofeca richardsi	AF070445	Mesomycetocoa
Ichtyophon hoferii	AY082999	Mesomycetocoa
Amphibiocystidium ranae	AY692319	Mesomycetocoa
Sphaeroforma arctica	Y16260	Mesomycetocoa
Corallochytrium limacisporum	L42528	Chorallochytrea
Pichia anomala	EF514696	Ascomycota
Ophiosphaerella herpotricha	DQ767650	Ascomycota
Phymatotrichopsis omnivora	EF441992	Ascomycota
Botrytis tulipae	AM233399	Ascomycota
Saitoella complicata	AY548297	Ascomycota
Candida sp.	DQ177817	Ascomycota
Botryomyces caespitosus	Y18695	Ascomycota
Pileolaria toxicodendri	DQ092921	Basidiomycota
Stemonitis axifera	AY145528	Mycetozoa
Dictyostelium firmibasis	AY040330	Mycetozoa
Didymium nigripes	AY223840	Mycetozoa
Hemitrichia serpulae	AY223841	Mycetozoa

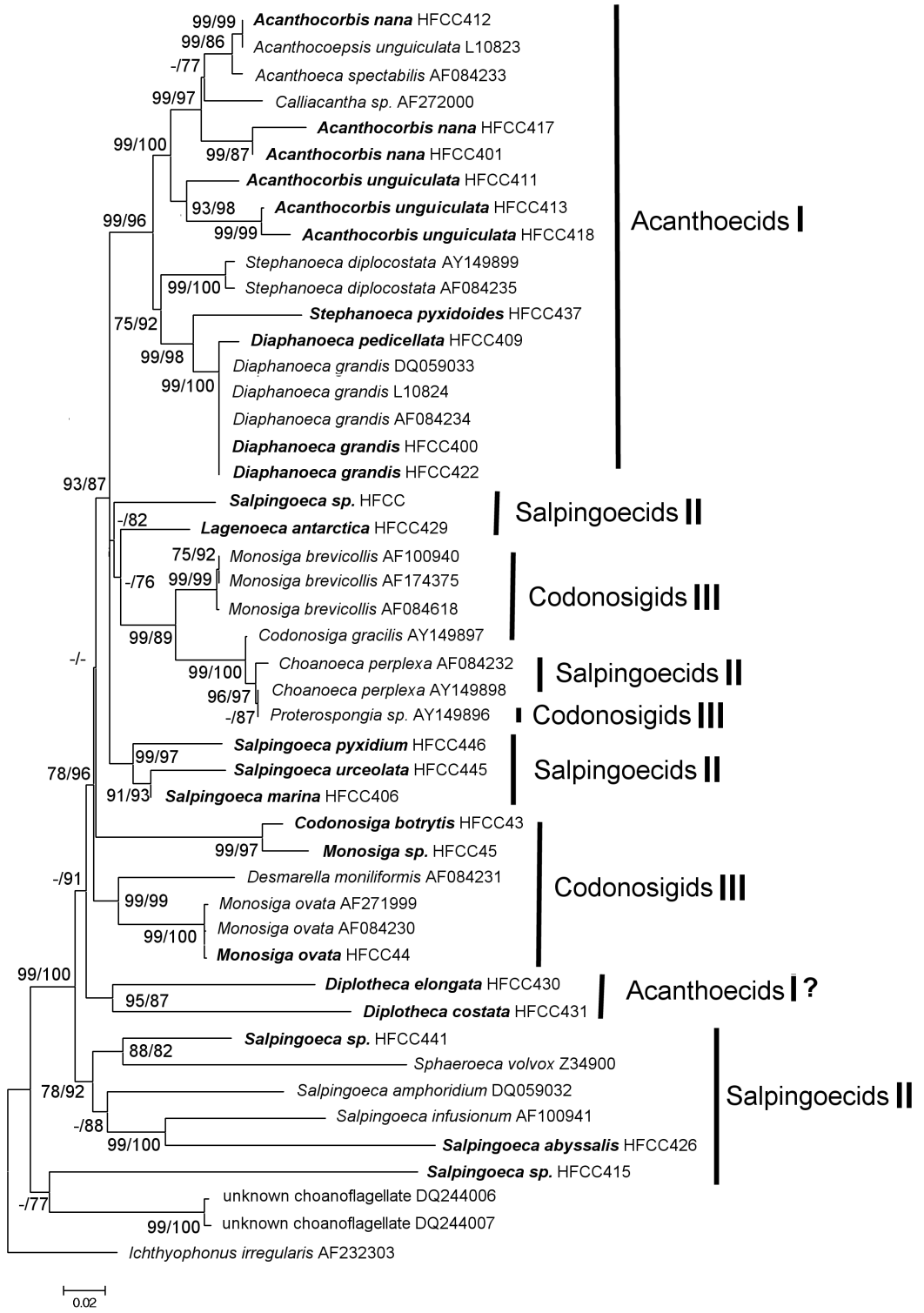

Figure 3. Phylogenetic bootstrap analysis of partial SSU rRNA from choanoflagellate sequences listed in Table 2. First value for NJ, second for ML (see methods). Missing numbers indicate values under 75%.

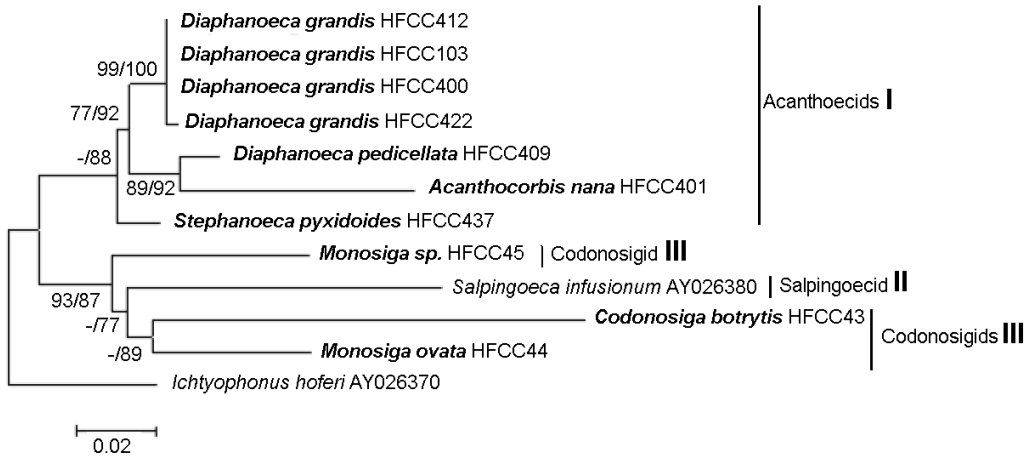

Figure 4. Phylogenetic bootstrap analysis of partial LSU rRNA from choanoflagellate sequences listed in Table 2. First value for NJ, second for ML (see methods). Missing numbers indicate values under 75%.

Discussion

An important result of the present investigation was that the families of salpingoecid and codonosigid choanoflagellates will have to be reassigned as they do not show any consistency on the molecular biological level. Although the family of acanthoecid choanoflagellates formed a consistent cluster in our SSU rRNA bootstrap analysis, the cluster was divided into two subclusters.

Morphological examinations of the lorica formation during cell division (Leadbeater pers. comm.) confirmed that there are two lorica types within acanthoecids. This hypothesis is reflected in the formation of the two subclusters which were confirmed by the analysis of other genes (Leadbeater pers. com.). The absence of *Diplotheca elongata* and *D. costata* in the acanthoecid cluster is supported morphologically by their unique lorica structure. Instead of the costal rods typical for acanthoecids their lorica consists of flattened costal strips (Nitsche & Arndt subm.). This might indicate an early separation of acanthoecid choanoflagellates.

With the up to now largest dataset of choanoflagellate SSU rRNA sequences we also examined the position of choanoflagellates within the Opisthokonta. Our single-gene analysis supported the hypothesis of a monophyletic origin of metazoans. The branching of the tree confirmed the hypothesis that Choanozoa and Fungi form a sister group to metazoans. In future, it would be necessary to sequence further genes of choanoflagellates to test this hypothesis. The hypothesis that Mesomycetozoa are the closest related protists to Metazoa (Medina *et al*. 2001) could not be confirmed. Despite recent advances, much controversy remains about the exact position of these groups at the animal fungal divergence.

Literature

Atkins, M. S., McArthur, A. G. and Teske, A. P. (2000) Ancyromonadida: a new phylogenetic linege among the protozoan closely related to the common ancestor of metazoans, fungi and choanoflagellates (Ophistokonta). *J. Mol. Evol.*, **51**: 278-285.

Ax, P. (1989) Basic phylogenetic systematization of the Metazoa.In: The Hierarchy of Life. Fernholm B & JörnwallH, eds., pp. 229–245. Elsevier, Amsterdam.

Baker, G. C., Beebee, T. J. C. and Ragan, M. A. (1999) *Prototheca richardsi*, a pathogen of anuran larvae, is related to a clade of Protistan parasites near the animal-fungal divergence. *Microbiol.* **145**: 1777-1784

Baldauf, S. L., and Palmer, J. D. (1993). Animals and fungi are each other's closest relatives: congruent evidence from multiple proteins. *Proc. Natl. Acad. Sci. USA* **90**: 11558–11562.

Baldauf, S. L., Roger, A. J., Wenk-Siefert, I., and Doolittle, W. F. (2000). A kingdom-level phylogeny of eukaryotes based on commbined protein data. *Science* **290**: 972–977.

Baldauf, S. L. (2003) The deep roots of eukaryotes. Science, **300**: 1703-1706.

Barnes, R. D. (1985) Current perspectives on the origins and relationships of lower invertebrates. In: The Origins and Relationships of Lower Invertebrates. Conway-Morris S, George JD, Gibson R, & Platt HM, eds., pp. 360–367. Clarendon Press, Oxford.

Cavalier-Smith, T. & Chao, E. E-Y. (2003) Phylogeny of Choanozoa, Apusozoa, and other Protozoa and early eukaryote megaevolution. *J. Mol. Evol.* **56**: 540–563.

Clark, H. (1868) On the Spongiae ciliatae as *Infusoria flagellata,* or observations on the structure, animality and relationship of *Leucosolenia botryoides* Bowerbank. *Ann. Mag. Nat. Hist*. **4**: 133–264.

Guindon, S. & Gascuel, O. (2003) A simple, fast and accurate algorithm to estimate large phylogenies by Maximum Likelihood*. Syst. Biol.* **52**: 696-704.

Kumar, S., Tamura, K., Nei, N. (2004) MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. *Briefings in Bioinformatics* **5**: 50-163.

Haeckel, E. (1874) Die Gastraea-Theorie, die phylogenetische Classification des Thierreichs und die Homologie der Keimblätter. *Z. Naturw*. **8**: 1–55.

Kent, S. (1878) Notes on the embryology of sponges. Ann. Mag. Nat. Hist. **5**: 139–156.

Kumar, S., & Rzhetsky, A, (1996) Evolutionary relationships of eukaryotic kingdoms. *J. Mol. Evol.* **42**: 183–193.

Lang, B. F., O'Kelly, C., Nerad, T., & Gray, M. W. (2002) The closest unicellular relatives of animals. *Curr. Biol*. **12**: 1773– 1778.

Maldonado, M. (2004) Choanoflagellates, choanocystes and animal multicellularity. Invert. Biol. **123**: 1-22.

Medina, M., Collins, A. G., Silberman, J. D. and Sogin, M. L.(2001) Evaluating hypotheses of basal animal phylogeny using complete sequences of large and small subunit rRNA. *Proc. Natl. Acad. Sci. USA* **98**: 9707–9712.

Nitsche, F. & Arndt, H. (subm.) A new choanoflagellate species from Taiwan: Morphological and molecular biological studies of *Diplotheca elongata* nov. spec. and *D. costata.* Europ. J. Prot.

Nitsche, F. & Arndt, H. (unpub.) Bipolar comparisons of acanthoecid choanoflagellates.

Nylander, J. A. A. (2004) MrAIC.pl. Program distributed by the author. Evolutionary Biology Centre, Uppsala University, Uppsala.

Ragan, M. A, Goggin, C. L., Cawthorn, R. J., Cerenius, L., Jamieson, A. V. C., Plourde, S. M., Rand, T. G., Soderhall, K., & Gutell, R. (1996) A novel clade of protistan parasites near the animal fungal divergence*. Proc. Natl. Acad. Sci*. *USA* **93**: 11907–11912.

Ruiz-Trillo, I., Lane, C.E., Archibald, J. M. and Roger, A. J. (2006) Insight into evolutionary origin and genome architecture of the unicellular ophistokonts *Capsaspora owczarzaki* and *Spaeroforma arctica*. J. Eukaryot. Microbiol., **53**: 379-384.

Salvini-Plawen, L. (1978) On the origin and evolution of the lower Metazoa. Z. Zool. Syst. Evolutionsforsch. **16**: 40–88.

Schulze, F. E. (1885) Über das Verhältnis der Spongien zu den Choanoflagellaten. *Sitzungsber. Königl. Preuss. Akad. Wiss* . **10**: 1–13.

Thompson, J. D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools*. Nucleic Acids Rese*arch **24**: 4876-4882.

Wainright, P. O., Hinkle, G., Sogin, M.L. and Stickel, S.K. (1993) Monophyletic origins of the metazoans: an evolutionary link with fungi. *Science* **260**: 340–342.

ABSTRACT

The global distribution of heterotrophic nanoflagellates is still heavily disputed. One part of the discussion consideres the diversity within protists. There are far less species than should be a priori expected by the log-normal species-body-length relationship (May 1999). For more than two centuries, morphology has been the standard of identification and taxonomy, but especially within heterotrophic nanoflagellates (HNF) the identification based on morphology meets its limits due to the lack of distinguishable features (Schlegel & Meisterfeld 2003). As a high number of cryptic species seems to be present within many morphospecies (Sáez & Lozano 2005), one may assume that morphologically defined species of protists are just a facade behind which lurk a high number of cryptic species (Cairns 1993). Current studies, implementing molecular biology, showed that there is a speciation going on, either allopatric or sympatric, resulting in a high number of these cryptic species (Darling *et al.* 2004; de Vargas *et al.* 1999; Nanney *et al*. 1998; Scheckenbach *et al*. 2005; 2006). In the discussion on the global distribution of protists, the species concept is an important issue.

The study on the biogeographic distribution of acanthoecid choanoflagellate morphospecies showed that the hypothesis "everything is everywhere – the environment selects" (Finlay 2004) was only limited applicable on acanthoecids. Out of 103 well defined morphospecies one third did indicate a limited biogeographic distribution. The polar regions with their comparable ecological conditions did show a very distinct species composition, separating the Arctic from the Antarctic.

The morphological and molecular biological comparison of the bipolar coldwater species *Acanthocorbis nana* and *A. unguiculata* revealed that even within these well defined morphospecies a number of cryptic species is hidden. In our examination of the SSU rRNA of seventy-four clonal sequences we found three genotypes within *A. nana*, not only suggesting an allopatric speciation between the polar regions but also a sympatry within the northern hemisphere. Another two genotypes were found in the morphospecies *A. unguiculata* varying between the northern and southern hemisphere. These results indicated that once contiguous populations were separated by the formation of the coldwater provinces in Miocene. The gene transfer seemed to be limited by the tropics. On the other hand our examination of a cosmopolitan species, *Diaphanoeca grandis*, did show, that there is little to no variance in the SSU rRNA, making it likely, that there is a gene transfer between the bipolar populations.

In addition, the finding of the three new species in a well defined group as choanoflagellates demonstrated that the number of protistan species may be underestimates. A large and morphologically distinct species like *Diplotheca elongata*, bearing the characteristics of a 'flagship species' in the sense of Foissner (1999) was found in the estuary of river Danshui in northern Taiwan. This species seemed to be limited to this particular region, suggesting that it has a restricted biogeographical distribution.

The other two new choanoflagellate species, *Lagenoeca antarctica* from the Weddell Sea and *Salpingoeca abyssalis* from the Cape Abyssal Plain were the first records of choanoflagellates from deep sea regions deeper than 2.500m. Deep-sea floor represents the largest part of the earth´s surface, making it likely that there is a high number of undescribed heterotrophic nanoflagellate species as choanoflagellates hidden in the abyssal.

The phylogenetic bootstrap analysis of the SSU and LSU rRNA from all studied choanoflagellates showed, that the salpingoecid and codonosigid choanoflagellates are polyphyletic. Within the family of acanthoecids there were found two branches supported by high bootstrap values. The clusters were containing species, varying in their formation of the lorica during cell division (pers. comm. by Leadbeater). Additionally, the analysis positioned the two species of the genus *Diplotheca*, morphologically assigned to the family of acanthoecids, significantly outside that cluster. This may indicate that the distinct lorica structure of this genus is a parallel evolution to the other lorica bearing choanoflagellates. These results showed that the taxonomy of choanoflagellates is in need of a fundamental.

The phylogenetic study of the position of Choanozoa within the Opisthokonta indicated that Fungi and Choanozoa form a sister group to the Metazoa, and supported the hypothesis of a monophyletic origin of metazoans.

The findings demonstrate that even morphologically distinguishable morpho-species like acanthoecid Choanoflagellates shelter a number of cryptic species, and thus the worldwide distribution of protists should be re-questioned. This indicates that protist diversity must be considered as grossly underestimated by morphology. The high intraspecific genetic divergence should not only be regarded as the result of neutral mutation as suggested by some authors (Fenchel & Finlay 2004). The systematic based on mere morphology is crumbling when molecular biological and ecological differences among nominal species are examined.

ZUSAMMENFASSUNG

Die globale Verbreitung von heterotrophen Nanaoflagellaten (HNF) ist mit eine der am meisten diskutierten Fragestellungen in der Protozoologie. Ein Aspekt dieser Diskussion ist die Diversität von Protisten. Grund für diese grundlegende Frage ist das Vorhandensein von weit aus weniger Arten als anhand des log-normal Verhältnisses von Artenzahl zu Individuengröße zu erwarten wäre (May 1999). Seit über 200 Jahren ist die Morphologie das ausschlaggebende Kriterium für die Bestimmung von Protisten. Aber besonders innerhalb der Gruppe der HNF stößt eine rein morphologisch basierte Taxonomie mangels erkennbarer Merkmale an ihre Grenzen (Schlegel & Meisterfeld 2003). Der hohe Anteil an morphologisch nicht identifizierbaren oder kryptischen Arten (Saez & Lozano 2005), legt die Vermutung nahe, dass hinter der Fassade dieser `Morpho-Arten´ eine gewaltige Menge an kryptischen Arten verborgen ist (Cairns 1993). Aktuelle molekular biologische Untersuchungen haben gezeigt, dass sowohl durch allopatrische als auch sympatrische Artbildung eine hohe Anzahl an kryptischen Arten entstanden ist (Darling *et al.* 2004; de Vargas *et al*. 1999; Nanney *et al*. 1998; Scheckenbach *et al*. 2005; 2006). In der Diskussion um die biogeographische Verbreitung von Protisten stellt das Artkonzept einen wichtigen Aspekt dar.

Anhand der Untersuchung der biogeographischen Verbreitung acanthoecider Choanoflagellaten konnte demonstriert werden, dass die Hypothese „everything is everywhere – the environment selects" (Finlay 2004) nur zum Teil auf die untersuchte Gruppe zutrifft. Von 103 Morpho-Arten zeigte ein Drittel eine begrenzte biogeographische Verbreitung wobei besonders die polaren Regionen hervorstachen. Trotz der vergleichbaren ökologischen Bedingungen zeigten die nördliche und die südliche polare Provinzen jeweils eine sehr charakteristische Artzusammensetztung.

Die morphologischen und molekular biologischen Untersuchungen an zwei bipolaren Kaltwasserarten, *Acanthocorbis nana* und *A. unguiculata*, zeigten deutlich, dass selbst innerhalb einer Gruppe mit wohl definierten morphologischen Charakteristika, eine Anzahl an kryptischen Arten verborgen ist. Die Untersuchung der SSU rRNA von 74 Klonen enthüllte drei Genotypen innerhalb der Morphoart *A. nana* und weitere zwei Genotypen in *A. unguiculata*. Dabei zeigte sich bei *A. nana* nicht nur ein Hinweis auf eine allopatrische Artbildung zwischen den Polen sondern auch eine Sympatrie innerhalb der nördlichen Hemisphäre. Innerhalb der Morphoart *A. unguiculata* zeigten sich ebenfalls unterschiedliche Genotypen, räumlich und zeitlich im Miozän durch die Tropen getrennt. Untersuchungen der SSU rRNA einer kosmopolitischen Art aus beiden polaren Regionen, *Diaphanoeca grandis*, zeigten

geringe bis keine Unterschiede im Genotypus und weisen auf einen kontinuierlichen Genfluß zwischen den Populationen hin.

Die Funde von drei neuen Choanoflagellatenarten zeigen, dass die Artenzahl der Protisten möglicherweise unterschätz wird. *Diplotheca elongata*, eine große und morphologisch signifikante Art aus der Flussmündung des Danshui in Nordtaiwan erfüllt die Bedingungen für die Bezeichnung Flaggschiff-Art im Sinne von Foissner (1999). Diese neue Art wurde bis jetzt nur in dieser speziellen Region gefunden, nicht aber in anderen Flussmündungen in Taiwan. Das legt den Schluss nahe, dass diese Art möglicherweise endemisch ist.

Mit der Beschreibung zweier weiterer neuer Arten wurden zum ersten Mal Choanoflagellaten aus der Tiefsee unterhalb von 2.500m nachgewiesen. *Lagenoeca antarctica* aus dem Abyssal des Weddell Meers und *Salpingoeca abyssalis* aus dem abyssalen Cap Becken. Die Tatsache, dass die Tiefsee den größten Teil der Erdoberfläche bedeckt, lässt darauf schließen, dass eine hohe Anzahl an HNF und damit auch Choanoflagellaten in der Tiefe verborgen sind.

Zur Untersuchung der Phylogenie innerhalb der Choanoflagellaten und ihrer phylogenetischen Position in der Gruppe der Opisthokonta wurden Sequenzen der SSU und LSU rRNA aller untersuchten Arten herangezogen. Die bootstrap Analyse zeigte, dass die codonosigiden und salpingoeciden Choanoflagellaten polyphyletisch sind. Innerhalb des Clusters der acanthoeciden Choanoflagellaten gibt es eine Trennung in zwei Gruppen, die morphologisch durch die Lorica-Bildung während der Zellteilung widergespiegelt wird (pers. Anm. Leadbeater). Mit zwei Ausnahmen spiegelte sich die taxonomische Einheit der acanthoeciden Familie in der Analyse wider. Die beiden Arten weisen eine sehr eigene Lorica-Struktur auf und die genetischen Distanzen zu den anderen lorica-tragendend Choanoflagellaten lässt vermuten, dass es sich um eine parallel Evolution handelt. Diese Ergebnisse zeigen, dass die Taxonomie der Choanoflagellaten grundlegend revidiert werden sollte.

Die phylogenetische Untersuchung der Position der Choanozoa innerhalb der Opisthokonta deutet darauf hin, dass die Choanozoa zusammen mit den Pilzen einer Schwestergruppe zu den Metazoa bilden. Weiters unterstützt sie die Hypothese, dass sie Metazoa monophyletisch sind.

Diese Untersuchung zeigt, dass selbst in einer morphologisch gut definierten Gruppe wie den acanthoeciden Choanoflagellaten eine vermutlich hohe Anzahl an kryptischen, zum Teil biogeographisch getrennter Arten, verborgen ist und damit auch die Hypothese der weltweiten Verbreitung von Protisten allgemein in Frage zu stellen ist. Dies bestätigt, dass die Diversität der Protisten durch ein rein morphologisches

Artkonzept stark unterschätzt wird. Die hohen intraspezifischen genetischen Unterschiede sollten nicht nur als ein Ergebnis neutraler Mutation, wie von Fenchel und Finlay (2004) interpretiert werden. Vielmehr zeigt sich, dass eine rein morphologisch basierte Systematik angesichts der Ergebnisse aus molekular biologischen und ökologischen Studien keinen Bestand haben kann.

LITERATUR

Cairns, J. J. (1993) Can microbial species with a cosmopolitan distribution become extinct? *Speculat. Sci. Technol.* **16**: 69–73.

Darling, K. F., Kucera, M., Pudsey, C. J., Wade, C. M. (2004) Molecular evidence links cryptic diversification in polar planktonic protists to Quaternary climate dynamics. *Proc. Natl. Acad. Sci*. **101**: 7657–7662.

de Vargas, C., Norris, R., Zaninetti, L., Gibb, S. W., Pawlowski J. (1999) Molecular evidence of cryptic speciation in planktonic foraminifers and their relation to oceanic provinces. *Proc. Natl. Acad. Sci. USA*. **96**: 2864-2868.

Fenchel, T., Finlay, B. J. (2004) Here and there or everywhere? Response from Fenchel and Finlay. *Bioscience* **54**: 884–885.

Finlay, B. J. (2004) Protist taxonomy: an ecological perspective. *Phi.l Trans. R. Soc.Lond., Ser B.* **359**: 599–610.

Foissner, W. (2006) Biogeography and dispersal of micro-organisms: A review emphasizing protists. *Act. Protozool.* **45**, 111-136.

May, R. M. (1999) Unanswered quetions in ecology. *Phil. Trans. R. Soc. London B.* **354**: 1951-1959.

Nanney, D. L., Park, C., Preparata, R., Simon, E. (1998) Comparison of sequence differences in a variable 23S rRNA domain among sats of cryptic species of ciliated protozoa. *J. Euk. Microbiol*. **45**: 91-100.

Sáez, A. G., Lozano, E. (2005) Body doubles. Cryptic species: as we discover more examples of species that are morphologically indistinguishable, we need to ask why and how they exist. *Nature* **433**:111.

Scheckenbach, F., Wylezich C., Weitere M., Hausmann K., Arndt H. (2005) Molecular identity of strains of heterotrophic flagellates isolated from surface waters and deep-sea sediments of the South Atlantic based on SSU rDNA. *Aquat. Microb. Ecol.* **38:** 239-247.

Scheckenbach, F., Wylezich C., Mylnikov A. P., Weitere M., Arndt H. (2006) Molecular comparisons of freshwater and marine isolates of the same morphospecies of heterotrophic flagellates. *Appl. Environ. Microbiol*. **72**: 6638-6643.

Schlegel, M., Meisterfeld R. (2003) The species problem in protozoa revisited. *Eur. J. Protistol.* **39**: 349–355.

ANHANG

Table S1: Table containing the presents/absents data of acanthoecid choanoflagellates in the listed bioregions. N indicating own findings.

Acanthoecid species	**1**	**2A**	**2P**	**3A**	**3P**	**4A**	**4P**	**5P**	**5I**	**6A**	**6P**	**6I**	**8**	**9**	**B**	**M**	**N**	**BS**	**Literature**
Acanthocorbis apoda (Leadbeater, 1972) Hara & Takahashi, 1984	1	1	1	1	1	0	1	0	1	0	1	0	0	0	1	1	1	0	30,32,33,46,54,59,62,63,70,77,80,83
Acanthocorbis assymetrica (Thomsen, 1979) Hara & Takahashi, 1984	0	0	1	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	35,48,62,70,83,87
Acanthocorbis campanula (Espeland, 1986) Thomsen in Thomsen et al., 1991	1	1	0	1	1	0	1	0	1	0	1	0	0	0	1	0	1	0	18,70,77,80
Acanthocorbis camarensis Hara etal 1996	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	20 ,26
Acanthocorbis haurakiana Thomsen in Thomsen et al., 1991	0	0	0	1	1	0	1	0	0	0	1	0	0	0	1	1	0	0	70,77,80
Acanthocorbis nana Thomsen et al.,1997	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	77, N
Acanthocorbis prolongata Thomsen et al.,1997	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	77
Acanthocorbis tinnabulum Marchant et al., 1987	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	49,70,76
Acanthocorbis unguiculata (Thomsen, 1973) Hara & Takahashi, 1984	1	1	1	1	1	0	0	0	0	0	0	0	0	1	1	0	1	0	10,12 ,19,20,47,49,55,64,67,76,70,77,82,83,86,N
Acanthocorbis weddellensis Thomsen et al., 1997	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	77
Acanthoeca brevipoda Ellis, 1930	0	0	1	1	0	0	0	0	0	0	1	0	0	1	1	0	1	0	9,5,19,34,49,54,64,70,76,82,85,86
Acanthoeca spectabilis Ellis, 1930	1	1	1	1	1	0	1	0	0	1	1	1	0	1	1	0	1	0	1,5,9,20,28,33,38,47,54,64,67,76,82,83,84,85,88,N
Amoenoscopa caudata Hara & Takahashi, 1987	0	0	0	0	1	0	1	0	0	0	0	0	0	0	1	0	0	0	21,70,75
Apheloecion antarctica Thomsenet al., 1997	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	77
Apheloecion articulatum Thomsen & Boonruang, 1983	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	70,72
Apheloecion conicoides Thomsen et al., 1997	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	77
Apheloecion glacialis Thomsen et al., 1997	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	77
Apheloecion pentacanthum Thomsen in Thomsen & Boonruang, 1983	0	0	0	0	1	0	1	0	1	0	0	0	1	0	0	0	0	0	28,70,72,75
Apheloecion quadrispinum Thomsen in Thomsen & Boonruang, 1983	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	70,72
Bicosta antennigera Moestrup, 1979	1	1	1	0	0	0	0	0	0	0	1	0	0	1	1	0	0	0	7,8,19,20,26,51,52,63,70,76,77,78
Bicosta minor (Reynolds, 1976) Leadbeater, 1978	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	1	0	24,51,52,55,58,68,70,76,82,84,85,88
Bicosta spinifera (Throndsen, 1970) Leadbeater, 1978	1	1	1	1	1	0	1	0	0	1	1	0	1	1	1	0	1	0	7,8,10,13,19,24,26,40,47,51,58,63,64,68,70,76,77,82,83,85
Calliacantha ankyra Thomsen et al., 1997	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	77
Calliacantha frigida Thomsen et al., 1997	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	77
Calliacantha longicaudata Leadbeater, 1978	1	1	1	1	1	0	0	0	0	0	0	0	0	1	1	0	0	0	12,46,51,68,70,77,82
Calliacantha multispina Manton & Oates, 1979	1	0	0	1	0	0	0	0	0	1	1	0	1	1	0	0	0	0	7,26,40,70,76,77,78,82,85
Calliacantha natans (Grøntved, 1956) Leadbeater, 1978	1	1	1	1	1	0	0	0	1	0	1	0	1	1	1	1	0	0	8,10,26,34,40,47,51,52,62,63,64,66,67,68,70,76,82,83,85,88
Calliacantha simplex Manton & Oates, 1979	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	0	0	7,8,10,19,26,40,44,47,61,67,68,70,75,76,82,83,84,85,88,51
Calotheca alata Thomsen & Moestrup, 1983	0	0	0	0	1	0	0	0	1	0	1	1	1	0	0	0	0	0	70,79,84,85
Campyloacantha imbricata Hara & Takahashi, 1987	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	25,28,70
Campyloacantha spinifera (Leadbeater, 1973) Hara & Takahashi, 1987	0	0	0	0	1	1	1	0	0	0	1	1	1	0	0	1	0	0	25,35,70,75,84,85
Conion groenlandicum Thomsen, 1982	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	68,70

Cosmoeca ceratophora Thomsen in Thomsen & Boonruang, 1984	0	0	0	0	1	1	1	0	1	0	1	0	0	0	0	1	0	0	70,73
Cosmoeca norvegica Thomsen in Thomsen & Boonruang., 1984	1	1	1	1	1	1	0	0	1	0	1	0	1	1	1	1	1	0	20,40,70,73,76,82,85
Cosmoeca phuketiensis Thomsen in Thomsen & Boonruang, 1984	0	0	0	0	1	1	1	0	1	0	1	1	0	0	0	1	0	0	28,70,73,84,85
Cosmoeca subulata Thomsen, 1984	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	73
Cosmoeca takahashii Thomsen,1990	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	40,76,78
Cosmoeca ventricosa Thomsen in Thomsen & Boonruang., 1984	1	1	1	1	1	0	1	0	1	0	1	1	1	1	1	1	1	0	8,12,19,28,35,36,40,47,63,65,67,68,70,73,75,76,78,83,84,85,88
Crinolina aperta Thomsen, 1976	1	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	7,8,10,13,19,20,26,40,47,63,70,76,77,78,83
Crinolina isefiordensis Thomsen, 1976	1	1	0	1	1	0	1	0	1	0	1	0	1	0	1	1	1	0	28,52,65,68,70,82,85
Crucispina cruciformis (Leadbeater, 1974) Espeland in Espeland & Throndsen, 1986	0	0	0	1	0	0	1	0	0	0	1	1	0	0	0	1	0	0	24,36,52,66,75,82,84,85
Diaphanoec aperta Manton et al, 1975	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	27,46
Diaphanoeca cylindrica Leadbeater, 1974	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	36
Diaphanoeca grandis Ellis, 1930	1	1	1	1	1	1	0	0	1	0	1	1	0	1	1	1	1	1	2,9,5,12,19,33,35,36,43,47,48,49,55,64,67,68,70,76,78,80,81,82,83,84,85,86,87,88,89,90,N
Diaphanoeca multiannulata Buck, 1980	1	1	0	0	0	0	0	0	0	0	1	0	1	1	0	0	0	0	1,7,8,10,19,20,26,40,47,63,70,76,77,78
Diaphanoeca pedicellata Leadbeater, 1972	1	1	1	1	1	0	1	0	0	0	1	0	1	1	1	1	0	0	8,10,19,26,34,36,40,47,51,64,65,67,68,70,76,82
Diaphanoeca sphaerica Thomsen, 1982	1	1	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	0	12,20,29,70,76,88
Diaphanoeca spiralifurca Hara et al., 1996	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	22,28
Diaphanoeca undulata Thomsen, 1982	1	1	1	1	1	1	1	0	0	0	1	1	0	0	1	0	1	0	28,68,70,75,82,84,85
Diplotheca costata Valkanov, 1970	0	0	0	1	0	0	1	0	1	0	1	0	0	0	1	1	1	1	28,30,34,35,36,64,67,70,82,84,87,N
Diplotheca elongata Nitsche&Arndt 2007	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	N
Kakoeca antarctica Buck and Marchant in Buck et al., 1990	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	9,19
Monocosta fennica Thomsen, 1979	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	29,67,88
Nannoeca minuta (Leadbeater, 1972) Thomsen, 1988	1	1	1	1	0	1	1	0	1	0	1	1	1	0	1	1	0	0	20,28,66,69,70,82,84
Parvicorbicula aculeatus Tong, 1997	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	82
Parvicorbicula circularis Thomsen, 1976	1	1	1	1	1	0	1	0	0	0	1	1	1	1	1	1	0	0	10,40,47,65,67,75,76,82,83,84,85,88
Parvicorbicula corynocostata Thomsen et al., 1997	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	77
Parvicorbicula manubriata Tong, 1997	1	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	1	0	77,82
Parvicorbicula ongulensis Takahashi, 1981	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	26,63,76
Parvicorbicula pachycostata Thomsen et al., 1997	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	77
Parvicorbicula pedicellata Leadbeater, 1973	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	1	0	0	35,36,70,85
Parvicorbicula pedunculata Leadbeater, 1980	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	39
Parvicorbicula quadricostata Throndsen, 1970	1	1	1	1	1	0	1	0	0	0	0	0	0	1	1	0	1	0	8,19,45,51,68,70,75,76,81,82
Parvicorbicula serrulata Leadbeater in Manton et al., 1975	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	46,68,70
Parvicorbicula socialis (Meunier, 1910) Deflandre, 1960	1	1	1	1	1	0	0	0	1	0	1	0	1	1	1	1	0	0	7,8,10,12,13,26,27,33,40,45,47,51,57,59,61,62,63,64,66,68,70,76,81,82,83,N
Parvicorbicula superpositus Booth, 1990	0	0	1	1	1	0	1	0	0	0	1	0	1	0	1	0	1	0	28,70,75,82,85
Parvicorbicula zigzag Thomsen et al., 1991	0	0	0	0	1	1	1	0	0	0	0	0	1	0	0	0	0	0	70,75
Platypleura acuta Thomsen & Boonruang, 1983	0	0	0	0	1	0	1	1	1	0	1	0	0	0	0	1	0	0	70,71,75
Platypleura cercophora Thomsen & Boonruang, 1983	0	0	0	0	0	0	1	0	1	0	0	1	0	0	0	0	0	0	28,71,84

Platypleura infundibuliformis (Leadbeater, 1974)Thomsen in Thomsen&Boonruang, 1983	0	0	0	0	0	1	1	0	1	0	1	0	1	0	1	1	1	0	28,36,66,70,71
Platypleura perforata Thomsen & Boonruang, 1983	0	0	1	0	1	0	1	0	1	0	1	0	0	0	0	0	0	0	70,71,75,85
Pleurasiga echinocostata Espeland in Espeland & Throndsen, 1986	0	0	1	0	1	1	1	0	1	0	1	0	1	0	1	1	1	0	28,40,70,75,85
Pleurasiga minima Throndsen, 1970	1	1	1	1	1	1	1	0	1	0	1	1	1	1	1	1	1	0	8,34,35,36,40,45,64,65,66,68,75,76,78,81,82,83,84,85
Pleurasiga reynoldsii Throndsen, 1970	1	1	1	1	1	0	1	0	0	0	1	1	1	1	1	1	0	0	35,45,47,52,62,64,65,70,75,76,78,81,82,84,85
Pleurasiga tricaudata Booth, 1990	0	0	1	0	1	0	1	0	0	0	1	0	1	0	0	0	0	0	70,75,85
Polyfibula elatensis Manton & Bremer,1981	1	0	1	0	1	0	1	0	1	0	1	0	1	0	0	0	0	0	70,75,85
Polyfibula sphyrelata (Thomsen, 1973) Manton in Manton & Bremer, 1981	1	1	1	1	1	0	1	0	0	1	1	0	1	1	1	1	0	0	46,47,52,62,64,66,67,68,70,75,76,82,85
Polyoeca dichotoma Kent, 1881	0	0	1	1	1	0	1	0	0	0	0	1	0	1	1	0	1	0	5,7,23,28,70,82,84
Saepicula leadbeateri Takahashi, 1981	1	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	20,26,47,63,76,77,78,83
Saepicula pulchra Leadbeater, 1980	0	1	0	1	0	0	0	0	0	0	0	1	0	0	1	0	0	0	39,70,82,84
Saroeca attenuata Thomsen, 1979	1	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	29,67,68,70,78,88
Saroeca paucicostata Hara & Takahashi 1987	0	1	0	0	1	0	1	0	0	0	1	0	0	0	0	1	0	0	25,28,70
Savillea micropora (Norris, 1965) Leadbeater, 1975	0	0	1	1	1	1	0	0	0	0	0	0	0	0	1	0	1	0	29,37,54,82,88,90
Savillea parva (Ellis, 1930) Loeblich III, 1967	0	0	1	1	1	0	1	0	0	0	0	0	0	1	1	0	1	0	5,19,27,28,34,54,64,76,82,88,N
Spiraloecion didymocostatum Marchant & Perrin 1986	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	48,76
Stephanacantha campaniformis (Leadbeater, 1973)Thomsen in Thomsen&Boonruang, 1983	0	0	1	0	1	0	1	0	1	0	0	0	1	0	0	1	1	0	35,36,66,70,71,75
Stephanoeca ampulla (Kent, 1880) Ellis, 1930	0	1	0	1	0	0	0	0	0	0	0	0	0	0	1	0	1	0	9,70,80
Stephanoeca apheles Thomsen, Buck & Chavez, 1991	0	0	0	0	1	0	1	0	0	0	1	0	0	0	1	1	0	0	28,70,75,85,88
Stephanoeca campanula (Kent, 1880) Boucard Camou, 1967	0	0	0	1	1	0	1	0	0	0	0	0	0	0	0	0	1	0	9,70,75
Stephanoeca cauliculata Leadbeater, 1980	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	3970,
Stephanoeca complexa (Norris, 1965) Throndsen, 1974	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	48,49,54,62,70,76
Stephanoeca constricta Ellis, 1930	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	1	0	9,70
Stephanoeca cupula (Leadbeater, 1972) Thomsen, 1988	1	1	0	1	0	0	1	0	0	0	1	0	0	1	1	0	0	0	20,27,28,64,69,70,76,82,85
Stephanoeca deminutiva (Norris, 1965) Throndsen, 1974	0	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	54,70
Stephanoeca diplocostata Ellis, 1930	1	1	0	1	1	0	1	1	0	0	1	1	1	1	1	1	1	0	9,28,29,32,33,34,36,37,51,64,68,70,75,80,82,84,86
Stephanoeca paucicostata Throndsen, 1969	1	1	0	0	1	1	1	0	1	0	1	1	0	1	1	1	1	0	19,28,47,49,64,67,68,70,75,76,80,83,84,85,88,89
Stephanoeca elegans (Norris, 1965) Throndsen, 1974	0	1	1	1	1	0	1	0	1	0	0	1	0	1	1	1	0	0	33,35,36,54,64,70,75,84
Stephanoeca kentii Ellis, 1930	0	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	9,54,80
Stephanoeca norrisii Thomsen, 1973	0	0	1	1	1	0	0	0	0	0	0	0	0	1	1	0	0	0	49,64,70,75,82
Stephanoeca pyxidoides Leadbeater, 1980	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	3970,
Stephanoeca supracostata Hara et al., 1996	0	0	0	1	0	0	1	0	0	0	0	0	0	1	0	0	0	0	22,28,70,82
Stephanoeca urnula Thomsen, 1973	0	0	1	1	0	0	0	0	0	0	0	0	0	0	1	1	0	0	29,64,67,70,88
Syndetophyllum pulchellum (Leadbeater, 1974) Thomsen & Moestrup, 1983	0	0	0	0	1	0	1	0	1	0	1	0	0	0	0	1	0	0	28,36,53,66,70,79,85

Table S2: List of publications used for the analysis of the biogeographic distribution of acanthoecid choanoflagellates.

References

1 Al-Quassab, S., Lee, W. J., Murray, S., Simpson, A. G. B. & Patterson, D. J. (2002). Flagellates from stromatolites and surrounding sediments in Shark Bay, Western Australia. *Acta Protozoologica*, **41**, 91-144.

2 Andersen, P. (1988/1989). Functional biology of the Choanoflagellate *Diaphanoeca grandis* Ellis. *Marine Microbial Food Webs*, **3**, 35-50.

3 Booth, B.C. (1990). Choanoflagellates from the sub arctic North Pacific Ocean, with descriptions of two new species. *Canadian Journal of Zoology*, **68**, 2393-2402.

5 Boucaud-Camou, E. (1967). Les choanoflagelles des cotes de la manche: I. Systematique. *Bulletin de la Société Linnéenne de Normandie*, **1966**, 191-209.

7 Buck, K.R. (1981). A study of choanoflagellates (Acanthoecidae) from the Weddell Sea, including a description of *Diaphanoeca multiannulata* n. sp. *Journal of Protozoology*, **28**, 47-54.

8 Buck, K.R. & Garrison, D.L. (1988). Distribution and abundance of choanoflagellates (Acanthoecidae) across the ice-edge zone in the Weddell Sea, Antarctica. *Marine Biology*, **98**, 263-269.

9 Buck, K.R., Marchant, H.J., Thomsen, H.A. & Garrison, D.L. (1991). Kakoeca Antarctica gen. et sp.n., a loricate choanoflagellate (Acanthoecidae, Choanoflagellida) from Antarctic sea ice with a unique protoplast suspensory membrane. *Zoologica Scribor*, **19**, 389-394.

10 Chen, B. (1994). Distribution and abundance of Choanoflagellates in Great-Wall Bay, King George Island, Antarctica in Austral summer. *Proceedings of the NIPR Symposium on Polar Biology*, **7**, 32-42.

12 Daugbjerg, N. & Vørs, N. (1992). Preliminary results from a small scale survey of marine protists from Northern Foxe basin in the vicinity of Igloolik Island June 1992. *Research on Arctic biology, Igloolik, Northwest territories, Canada.* (ed. By B. Søeberg, D. Jensen, H. Schurmann, J.F. Steffensen, M.A. Curtis, N. Vørs, N. Daugbjerg and P. Bushnell). Zoological Museum, University of Copenhagen.

13 Davidson, A.T., Marchant, H.J. (1992) Protist abundance and carbon concentration during a *Phaeocystis*-dominated bloom at an Antarctic costal site. *Polar Biology*, **12**, 387-395.

19 Garrison, D.L. & Buck, K.R. (1989). The biota of Antarctic pack ice in the Weddell Sea and Antarctic Peninsula regions. *Polar Biology*, **10**, 211-219.

20 Hansen, L., K. E. Johansen, T. Lund & Simonsen, B. (1989). Homoterme kilder på Disko, Grønland. *Feltkursus* (ed. M. Jørgensen, K. Johansen, T. Lund and B. Simonsen) pp.143–205. Arktisk Zoological Museum, University of Copenhagen: Biologie.

21 Hansson, H.G. (1997). South Scandinavian Marine Protoctista. http://www.tmbl.gu.se/libdb/taxon/neat_pdf

22 Hara, S., Lee Chen, Y.I., Sheu, J.C. & Takahashi, E. (1996). Choanoflagellates (Sarcomastigophora, Protozoa) from the coastal waters of Taiwan and Japan I. Three new species. *Journal of eukaryotic Microbiology* **43**, 136-143.
23 Hara, S.& Takahashi, E. (1984). Re-investigation of *Polyoeca dichotoma* and *Acanthoeca spectabilis* (Acanthoecidae: Choanoflagellida). *Journal of the Marine Biological Association of the United Kingdom*, **64**, 819-827.

24 Hara, S. & Takahashi, E. (1987). An investigation with electron microscope of marine choanoflagellates (Protozoa: Choanoflagellida) from Osaka Bay, Japan. I. Re-investigation of Bicosta spinifera, *B. minor* and *Crucispina cruciformis*. *Bulletin of the Plankton Society of Japan,* **34**, 1-13.

25 Hara, S. & Takahashi, E. (1987). An investigation with electron microscope of marine choanoflagellates (Protozoa: Choanoflagellida) from Osaka Bay, Japan. II. Two new genera and a new species of Acanthoecidae. *Bulletin of the Plankton Society of Japan,* **34**, 15-23.

26 Hara, S. & Tanoue, E. (1984). Choanoflagellates in the Antarctic Ocean, with special reference to *Parvicorbula socialis* (Meunier) Deflandre. *Memoirs of National Institute of Polar Research* (Special Issue), **32**, 1-13.

27 Hara, S., Tanoue, E., Zenimoto, M., Komaki, Y. & Takahashi, E. (1986). Morphology and distribution of heterotrophic protists along 75°E in the Southern ocean. *Memoirs of National Institute of Polar Research* (Special Issue), **40**, 69-80.

28 Hara, S., Sheu, J., Chen, Y.L. & Takahashi, E. (1996). Choanoflagellates (Sarcomastigophora, Protozoa) from the coastal waters of Taiwan and Japan II. Species composition and biogeography. *Zoological Studies*, **36**, 98-110.

29 Ikävalko, J. (1998). Further observations on flagellates within sea ice in northern Bothnian Bay, the Baltic Sea. *Polar Biology*, **19**, 323-329.

30 Jackson, S.M. & Leadbeater, B.S.C. (1991). Costal strip accumulation in the marine choanoflagellate *Diplotheca costata* Valkanov. *Journal of Protozoology*, **38**, 97-104.

32 Larsen, J. & Patterson, D.J. (1990). Some flagellates (Protista) from tropical marine sediments. *Journal of natural History*, **24**, 801-937.

33 Leadbeater, B.S.C. (1972). Ultrastructural observations on some marine choanoflagellates from the coast of Norway. *Journal of the Marine Biological Association of the United Kingdom,* **52**, 67-79.

34 Leadbeater, B.S.C. (1972). Fine structural observations on some marine choanoflagellates from the coast of Denmark. *British Phycological Journal*, **7**, 195-211.

35 Leadbeater, B.S.C. (1973). External morphology of some marine choanoflagellates from the coast of Jugoslavia. *Archiv für Protistenkunde*, **115**,

234-252.
36 Leadbeater, B.S.C. (1974). Ultrastructural observations on nanoplankton collected from the coast of Yugoslavia and the Bay of Algiers. *Journal of the Marine Biological Association of the United Kingdom,* **54**, 179-196.

37 Leadbeater, B.S.C. (1975). A microscopical study of the marine choanoflagellate *Savillea micropora* (Norris) comb. nov., and preliminary observations on lorica development in *S. micropora* and *Stephanoeca diplocostata* Ellis. *Protoplasma*, **83**, 111-129.

38 Leadbeater, B.S.C. (1979). Developmental and ultrastructural observations on two stalked marine choanoflagellates, *Acanthoecopsis spiculifera* Norris and *Acanthoeca spectabilis* Ellis. *Proceedings of the Royal Society of London (Ser B)*, **204**, 57-66.

39 Leadbeater, B.S.C. (1980). Four new species of loricate choanoflagellates from South Brittany, France. *Cahiers de Biologie Marine*, **21**, 345-353.

40 Leakey, R.J.G., Leadbeater, B.S.C., Mitchell, E., McCready, S.M.M. & Murray, W.A. (2002). The abundance and biomass of choanoflagellates and other nanoflagellates in waters of contrasting temperature to the north-west of South Georgia in Southern Ocean. *European Journal of Protistology*, **38**, 333-350.

41 Lee, W.J. & Patterson, D.J. (1998). Diversity and geographic distribution of free-living heterotrophic flagellates - analysis by PRIMER *Protist*, **149**, 229-243.

42 Lee, W.J. & Patterson, D.J. (2000). Heterotrophic flagellates (Protista) from marine sediments of Botany Bay, Australia. *Journal of Natural History*, **34**, 483-562.

43 Manton, I., Bremer, G. & Oates, K. (1981). Problems of structure and biology in a large collared flagellate (*Diaphanoeca grandis* Ellis) from arctic seas. *Proceedings of the Royal Society of London (Ser B)*, **213**, 15-26.

44 Manton, I. & Oates, K. (1979). Further observations on *Calliacantha* Leadbeater (Choanoflagellata), with special reference to *C. simplex* sp. nov. from many parts of the world. *Proceedings of the Royal Society of London (Ser B)*, **204**, 287-300.

45 Manton, I., Sutherland, J. & Leadbeater, B.S.C. (1976). Further observations on the fine structure of marine collared flagellates (Choanoflagellata) from arctic Canada and west Greenland: species of *Parvicorbicula* and *Pleurasiga*. *Canadian Journal of Botany*, **54**, 1932-1955.

46 Manton, I., Sutherland, J. & Leadbeater, B.S.C. (1975). Four new species of choanoflagellates from arctic Canada. *Proceedings of the Royal Society of London*, **189**, 15-27.

47 Marchant, H.J. (1985). Choanoflagellates in the Antarctic marine food chain. *Antarctic nutrient cycles and food webs* (ed. W.R. Siegfried, P.R. Condy and R.M. Laws), pp 217-276. Springer-Verlag, Berlin.

48 Marchant, H.J. & Perrin, R.A. (1986). Planktonic choanoflagellates from two Antarctic lakes including the description of *Spiraloecion didymocostatum* gen. et

sp. nov. *Polar Biology*, **5**, 207-210.
49 Marchant, H.J., Van den Hoff, J. & Burton, H.R. (1987). Loricate Choanoflagellates from Ellis Fjord, Antarctica including the description of *Acanthocorbis tintinnabulum* sp. nov. *Proceedings of the NIPR Symposium on Polar Biology*, **1**, 10-22.

51 McKenzie, C.H., Deibel, D., Thompson, R.J., MacDonald, B.A. & Penney, R.W. (1997). Distribution and abundance of choanoflagellates (Acanthoecidae) in the costal cold ocean of Newfoundland, Canada. *Marine Biology*, **129**, 407-416

52 Moestrup, Ø. (1979). Identification by electron microscopy of marine nanoplankton from New Zealand, including the description of four new species. New *Zealand Journal of Botany*, **17**, 61-95.
53 Montresor, M., Lovejoy, C., Orsini, L., Procaccini, G. & Roy, S. (2003). Bipolar distribution of the cyst-forming dinoflagellate *Polarella glacialis*. *Polar Biology*, **26**, 186-194.

54 Norris, E.R. (1965). Neustonic marine craspedomonadales (Choanoflagellates) from Washington and California. *Journal of Protozoology*, **12**, 589-602.

55 Patterson, D.J., Nygaard, K., Steinberg, G. & Turely, C.M. (1993). Heterotrophic flagellates and other protists associated with oceanic detritus throughout the water column in the mid North Atlantic. *Journal of the Marine Biological Association of the United Kingdom,* **73**, 67-95.

56 Patterson, D.J. & Simpson, A.G.B. (1996). Heterotrophic flagellates from coastal marine and hypersaline sediments in Western Australia. *European Journal of Protistology*, **32**, 423-448.

57 Rat'kova, T.N. & Wassmann, P. (2002). Seasonal variation and spatial distribution of phyto- and protozooplankton in the central Barents Sea. *Journal of Marine Systems*, **28**, 47-75.

58 Reynolds, N. (1976) Observations on *Salpingoeca spinifera* Throndsen and *S. minor* sp. nov. (Craspedophycea). *British Phycological Journal*, **11**, 13-17.

59 Riedel, A., Michel, C., Poulin, M. & Lessard, S. (2001) Taxonomy and Abundance of Microalgae and Protists at a First-Year Sea Ice Station near Resolute Bay, Nunavut, Spring to Early Summer 2001 *Canadian Data Report of Hydrography and Ocean Sciences*, **159**, 3-60.

60 Sherr, E.B. & Sherr, B.F. (1992) Trophic roles of pelagic protists: phagotrophic flagellates as herbivores. *Archiv für Hydrobiologie (Beih Ergebn Limnol)*, **37**, 165-172.

61 Silver, M.W., Mitchell, J.G. & Ringo, D.L. (1980) Siliceous Nanoplankton. II. Newly discovered cysts and abundant choanoflagellates from the Weddell Sea, Antarctica. *Marine Biology*, **58**, 211-217.

62 Sukhanova, I.N. (2001) Choanoflagellida on the Southeastern Bering Sea Shelf. *Oceanology*, **41**, 227-231.

63 Takahashi, E. (1981) Loricate and scale-bearing protists from Lützow-Holm

Bay, Antarctica I. Species of Acanthoecidae and the Centrohelida found at a site selected on the fast ice. *Nankyoku Shiryô (Antarctic Records)*, **73**, 1–22.
64 Thomsen, H.A. (1973) Studies on marine choanoflagellates I. Silicified choanoflagellates of the Isefjord (Denmark). Ophelia, 12, 1-26

65 Thomsen, H.A. (1976) Studies on marine choanoflagellates. II. Fine structural observations on some silicified choanoflagellates from the Isefjord (Denmark), including the description of two new species. *Norwegian Journal of Botany*, **23**, 33-51.

66 Thomsen, H.A. (1978) Nanoplankton from the Gulf of Elat (=Gulf of Aquaba) with particular emphasis on choanoflagellates. *Israelian Journal of Zoology*, **27**, 34-44.

67 Thomsen, H.A. (1979) Electron microscopical observations on brackish-water nanoplankton from the Tvärminne area, S.W. coast of Finland. *Acta Botanica Fennica*, **10**, 11-37.

68 Thomsen, H.A. (1982) Planktonic choanoflagellates from Disko Bugt, West Greenland, with a survey of the marine nanoplankton of the area. *Meddr Grønland (Biosciences)*, **8**, 3-35.

69 Thomsen, H.A. (1988) An electron microscopical study of marine loricate choanoflagellates: *Nannoeca minuta* (Leadbeater) gen. et comb. n. and *Stephanoeca cupula* (Leadbeater) comb. n. *Zoologica Scripta*, **17**, 315-323.

70 Thomsen, H.A. (1992) Loricabærende Choanoflagellater (Kraveflagellater). *Plankton in Plankton i de indre danske farvande* (ed. H.A. Thomsen), pp 157-194. Havforskning fra Miljøstyrelsen, 11.

71 Thomsen, H.A. & Boonruang, P. (1983) A microscopical investigation of marine collared flagellates (Choanoflagellida) from the Andaman Sea, S.W. Thailand: species of *Stephanacantha* gen. nov. and *Platypleura* gen. nov. *Protistologica*, **19**, 193-214.

72 Thomsen, H.A. & Boonruang, P. (1983) Ultrastructural observations on marine choanoflagellates (Choanoflagellida, Acanthoecidae) from the coast of Thailand: species of *Apheloecion* gen. nov. *Journal of Plankton Research*, **5**, 739-753.

73 Thomsen, H.A. & Boonruang, P. (1984) A light and electron microscopical investigation of loricate choanoflagellates (Choanoflagellida, Acanthoecidae) from the Andaman Sea, S.W. Thailand and Denmark: species of *Cosmoeca* gen. n. *Zoologica Scripta*, **13**, 165-181.

74 Thomsen, H.A. & Buck, K.R. (1991) Choanoflagellate diversity with particular emphasis on the Acanthoecidae. *The biology of free-living heterotrophic flagellates.* (ed. By D.J. Patterson DJ and J. Larsen), pp 259-284. Clarendon Press, Oxford.

75 Thomsen, H.A., Buck, K.R. & Chavez, F.P. (1991) Choanoflagellates of the central Californian waters: taxonomy, morphology and species assemblages. *Ophelia*, **33**, 131-164.

76 Thomsen, H.A., Buck, K.R., Coale, S.L., Garrison, D.L. & Gowing, M.M. (1990)

Loricate choanoflagellates (Acanthoecidae, Choanoflagellida) from the Weddell Sea, Antarctica. *Zoologica Scripta*, **19**, 367-387.
77 Thomsen, H.A., Garrison, D.L. & Kosman, C. (1997) Choanoflagellates (Acanthoecidae, Choanoflagellida) from the Weddell Sea, Antarctica, taxonomy and community structure with particular emphasis on the ice biota; with preliminary remarks on choanoflagellates from Arctic sea ice (Northeast Water Polynya, Greenland). *Archiv für Protistenkunde*, **148**, 77-114.

78 Thomsen, H.A. & Larsen, J. (1992) Loricate choanoflagellates of the Southern Ocean with new observations on cell division in *Bicosta spinifera* (Throndsen, 1970) from Antarctica and *Saroeca attenuata* Thomsen, 1979, from Baltic Sea. *Polar Biology*, **12**, 53-63.

79 Thomsen, H.A. & Moestrup, Ø. (1983) Electron microscopical investigations on two loricate choanoflagellates (Choanoflagellida), *Calotheca alata* gen. et. sp. nov. and *Syndetophyllum pulchellum* gen. et comb. nov., from Indo-Pacific localities *Proceedings of the Royal Society of London (Ser B)*, **219**, 41-52.

80 Throndsen, J. (1969) Flagellates of Norwegian coastal waters. *Nytt Magasin for Botanikk*, **16**, 161-216.

81 Throndsen, J. (1970) Marine Planktonic acanthoecaceans (Craspedophyceae) from arctic waters. *Nytt Magasin for Botanikk*, **17**, 103-111.

82 Tong, S.M. (1997) Choanoflagellates from Southampton Water, U.K., including the description of three new species. *Journal of the Marine Biological Association of the United Kingdom,* **77**, 929-958.

83 Tong, S.M. (1997) Heterotrophic flagellates and other protists from Southampton water, U.K. *Ophelia*, **47**, 71-131.

84 Tong, S.M. (1997) Heterotrophic flagellates from the water column in Shark Bay, Western Australia. *Marine Biology*, **128**, 517-536.

85 Tong, S.M., Nygaard, K., Bernard, C., Vørs, N. & Patterson, D.J. (1998) Heterotrophic flagellates from the water column in Port Jackson, Sydney, Australia. *European Journal of Protistology*, **34**, 162-194.

86 Tong, S.M., Vørs, N. & Patterson, D.J. (1997) Heterotrophic flagellates, centerhelid heliozoans and filose amoebae from marine and freshwater sites in the Antarctica. *Polar Biology*, **18**, 91-106.

87 Valkanov, A. (1970) Beitrag zur Kenntnis der Protozoen des Schwarzen Meeres. *Zoologischer Anzeiger*, **184**, 241-290.

88 Vørs, N. (1992) Heterotrophic amoebae, flagellates and heliozoa from the Tvärminne area, Gulf of Finland in 1988-1990. *Ophelia*, **36**, 1-109.

89 Vørs, N. (1993) Heterotrophic amoebae, flagellates and heliozoa from Arctic marine waters (North-West Territories, Canada and W. Greenland). *Polar Biology*, **13**, 113-126.

90 Vørs, N. (1993) Marine heterotrophic amoebae, flagellates and heliozoa from Belize (Central America) and Tenerife (Canary Islands), with descriptions of new

species, *Luffisphaera bulbochaete* N. Sp., *L. longihastis* N. Sp., *L. turriformis* N. Sp. and *Paulinella intermedia* N. Sp. *Journal of Eukaryotic Microbiology*, **40**, 272-287.

Köln, 20. April 2007

Erklärung

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.

Frank Nitsche

TEILPUBLIKATIONEN

- Nitsche, F., Weitere, M., Scheckenbach, F., Hausmann, K., Wylezich, C., Arndt, H., (2007) Deep sea records of choanoflagellates with a description of two new species. *Acta Protozool*. **46**
- Nitsche, F., Arndt, H., (subm.) Global biogeography of acanthoecid choanoflagellates (Eukaryota, Opisthokonta). *J. Biogeography*
- Nitsche, F., Arndt, H., (subm.) A new species of heterotrophic flagellates from Taiwanese brackish waters: Morphological and molecular biological studies of *Diplotheca elongata* nov. spec. and *D. costata* (Choanoflagellida, Acanthoecidae) *Europ. J. Protistol*
- Nitsche, F., Arndt, H., (subm.) Bipolar comparisons of acanthoecid choanoflagellates. *Appl. Env. Microbiol.*

CURRICULUM VITAE

FRANK NITSCHE

PERSÖNLICHE DATEN

Geburtsdatum: 23. Jannuar 1969

Geburtsort: Salzburg, Österreich

Staatsangehörigkeit: österreich

Geschlecht: männlich

SCHULBILDUNG

1975 - 1979 Grundschule Salzburg

1979 - 1987 Bundesgymnasium Salzburg

22. Juni 1987 Allgemeine Hochschulreife, Bundesgymnasium Salzburg

---

STUDIUM

1989 – 1996 Studium der Biologie an der Paris-Lodron-Universität Salzburg

23. Mai 1996 Magister im Fachgebiet Zoologie/ Biologie an der Paris-Lodron-Universität Salzburg.
Thema „Ökologie und Faunistik der Mollusken des Fuschlsees im besonderen von *Valvata piscinalis*"

2003 - 2007 Anfertigung der vorliegenden Dissertation unter der wissenschaftlichen Anleitung von Prof. Dr. Hartmut Arndt

---

BERUFLICHER WERDEGANG

1996 - 1998 Freiberufliche Arbeit als Biologe und Berater in Birmingham, England

1998 - 2001 Angestellter im Bereich IT, mit Schwerpunkt elektronische Datenbanken in Köln

2001 - 2003 Freiberufliche Arbeit als Programmierer und Berater in Köln