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Molecular identity of strains of heterotrophic flagellates isolated from surface waters and deep-sea sediments of the South Atlantic based on SSU rDNA

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ABSTRACT: Whereas much is known about the biodiversity of prokaryotes and macroorganisms in the deep sea, knowledge on the biodiversity of protists remains very limited. Molecular studies have changed our view of marine environments and have revealed an astonishing number of previously unknown eukaryotic organisms. Morphological findings have shown that at least some widely distributed nanoflagellates can also be found in the deep sea. Whether these flagellates have contact with populations from other habitats is still uncertain. We performed a molecular comparison of strains isolated from deep-sea sediments (>5000 m depth) and surface waters on the basis of their small subunit ribosomal DNA (SSU rDNA). Sequences of *Rhynchomonas nasuta*, *Amastigomonas debruynei*, *Ancyromonas sigmoides*, *Cafeteria roenbergensis* and *Caecitellus parvulus* were analysed, and 2 contrasting results obtained. Firstly, we found nearly identical genotypes within 1 morphospecies (*C. roenbergensis*), and secondly, quite different genotypes within certain morphospecies (*R. nasuta*, *A. sigmoides* and *C. parvulus*). In addition, high genetic distances between the different strains of *A. sigmoides* and *C. parvulus* indicate that these morphospecies should be divided into different at least genetically distinguishable species. In contrast, some heterotrophic nanoflagellates must indeed be regarded as being cosmopolitan. According to the low genetic distances between isolates of *R. nasuta*, *A. debruynei* and *C. roenbergensis* as well as between our isolates of *A. sigmoides* from deep-sea and surface waters, exchanges between these habitats and also on a global scale might be possible. In summary, our results show that 3 morphospecies obviously contain several cryptic species, while some of the investigated genotypes occur in both deep-sea as well as in surface waters.

KEY WORDS: Biodiversity · Deep sea · Heterotrophic flagellates · Molecular ecology · Phylogeny · Angola Basin · SSU rDNA

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INTRODUCTION

Heterotrophic flagellates are recognised as being fundamental components of aquatic ecosystems. Within planktonic and benthic food webs these microorganisms function as nutrient remineralisers and intermediaries to higher trophic levels. They are the primary consumers of bacteria, cyanobacteria and microalgae. Because of their high abundance, metabolic activity and their ability to ingest significant amounts of organic material, heterotrophic flagellates have been considered to be major nutrient recyclers in marine environments (Azam et al. 1983, Gasol & Vaqué 1993). The ecology of heterotrophic flagellates has been fairly well characterised; however, our knowledge of population structure and species diversity still remains quite limited (Arndt et al. 2000). Although many studies on species diversity of different locations of the marine littoral throughout the world have been carried out (e.g. Patterson & Simpson 1996,

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Ekelund & Patterson 1997, Tong 1997, Lee & Patterson 2000), little is known about the biogeography of most species, as many have only been reported to occur in a few locations. This might be an indicator for endemism, although studies on the community structure have not revealed a specific geographic distribution (Lee & Patterson 1998, Patterson & Lee 2000, Al-Qassab et al. 2002). The composition of flagellate communities in deep-sea environments and whether it is unique or not, is still unclear (Turley et al. 1988, Turley & Carstens 1991, Atkins et al. 1998, Hausmann et al. 2002, Arndt et al. 2003). Nearly all flagellates found in the deep sea have also been reported to occur in other locations (Patterson et al. 1993, Atkins et al. 2000, Patterson & Lee 2000, Arndt et al. 2003).

The geographic distribution of organisms is determined by their evolutionary history, their physiological preferences and by forces of dispersal (e.g. Fenchel et al. 1997). Small species with very high abundance and the possibility of forming resting stages (such as many flagellates) have a high probability of dispersal by (e.g.) global oceanic circulation, convective transport into the high strata of the atmosphere, or transport by animals such as birds (Finlay et al. 2001). Thus, large-scale dispersal across physical and geographical barriers may be possible and may have led to a global distribution (Finlay 2002). As ubiquity would limit the local speciation and extinction rate, the global number of species might be relatively small (Fenchel 1993). In contrast, our knowledge of the dispersal rates of microorganisms is very limited. Exchange rates between soil, groundwater and deep-sea habitats should be very low, although several morphospecies seem to occur in all 3 habitats (Arndt et al. 2003).

Most gaps in the available data on total number of species and their distribution are primarily the result of difficulties associated with identifying heterotrophic flagellates to the species level (Patterson & Lee 2000). Sufficient criteria for morphological taxonomic characterisation of flagellates can be obtained with electron microscopy (EM) (Foissner 1999), but even when molecular data indicate significant differences, EM studies may not always reveal significant morphological differences (A. P. Mylnikov pers. comm.). In addition, most field studies and species descriptions have been conducted using light microscopy, a method which may not be sufficiently discriminatory. Thus, it is possible that traditional morphospecies comprise a much greater number of ecologically or molecularly defined species. Recent studies based on small subunit ribosomal DNA (SSU rDNA) sequence data have revealed that morphospecies from different locations can be nearly genetically identical (Atkins et al. 2000, Darling et al. 2000). In contrast, some morphospecies of flagellated algae and ciliates (Proeschold et al. 2001) comprise groups clearly distinguishable by DNA comparison. Eukaryotic protistan diversity, at least in marine environments, seems to be much greater than presently assumed (López-García et al. 2001, Moon-van der Staay et al. 2001, Stoeck & Epstein 2003), but very little is known about the deep-sea benthic protists (Edgcomb et al. 2002).

Although the deep-sea floor represents the largest part of the earth's surface, its most abundant eukaryotic inhabitants have been little studied. In order to investigate the possible ubiquitous distribution of heterotrophic protists (Finlay 2002, 2004), we isolated flagellate strains from the Atlantic deep sea (South Atlantic, Angola Basin) from depths down to 5425 m. We were especially interested in the isolation of very commonly distributed species in order to determine whether these morphospecies can really be called cosmopolitan. We sequenced the SSU rDNA of *Rhynchomonas nasuta* Klebs, 1892, *Amastigomonas debruynei* De Saedeleer, 1931, *Ancyromonas sigmoides* Kent, 1880, and *Cafeteria roenbergensis* Fenchel & Patterson, 1988, and cf. *Caecitellus* (identified by light microscopy as *C. parvulus* Griessmann, 1913) (Patterson et al. 1993). These 5 morphospecies are widely distributed heterotrophic flagellates belonging to the 20 most common flagellate species world-wide (Patterson & Lee 2000). We compared the SSU rDNA from strains recovered from the deep sea with the SSU rDNA from strains recovered from surface waters.

MATERIALS AND METHODS

Organism collection. All species were collected in July 2000 during an expedition with RV 'Meteor' (Cruise 48, Leg 1; Expedition DIVA 1) in the oligotrophic South Atlantic, Angola Basin (a detailed overview is given in Table 1). They were collected from surface waters and from multicorer samples at depths between 5300 and 5500 m. Salinity was about 37 PSU and temperature was 17°C at the surface and 2.5°C in the deep sea. Plankton samples were taken from the surface by a water sampler. On deck, the samples were immediately filled into sterile 50 ml tissue flasks (Sarstedt). Benthos samples were taken by means of a multiple corer system (MUC). Only cores with undisturbed sediment and overlying water were sampled. The top and bottom of the corer were closed after sampling at the relevant depth. In addition, large sediment particles (max. 1 cm^3) were incubated for cultivation. The possibility of contamination during transport through the water column was checked with 'blind' samples (autoclaved material exposed in the sampling device during the whole

Table 1. Location and depth of collection of all species studied, with accession numbers for GenBank and Heterotrophic Flagellate Culture Collection Cologne (HFCC). Species sequenced in this study are in **bold**

Species	Accession no.	Sample location (depth)
Amastigomonas debruynei	HFCC48	Sediment, 18° 25.3′ S, 4° 44.0′ E, Angola Basin, South Atlantic Ocean (5392 m)
Amastigomonas debruynei	HFCC49	18° 19.5′ S, 4° 43.0′ E, Angola Basin, South Atlantic Ocean (2 m)
Amastigomonas debruynei	AY050180	Sargasso Sea, Atlantic Ocean (2500 m)
Ancyromonas sigmoides	HFCC60	Brackish water, Baltic Sea near Hiddensee, Germany (surface water)
Ancyromonas sigmoides	HFCC62	Sediment, 18° 19.5′ S, 4° 43.0′ E, Angola Basin, South Atlantic Ocean (5392 m)
Ancyromonas sigmoides	HFCC63	Sediment, 17° 11.6′ S, 4° 45.9′ E, Angola Basin, South Atlantic Ocean (5415 m)
Ancyromonas sigmoides	HFCC104	79° 07.27′ N, 4° 07.95′ E, North Atlantic Ocean (1804 m)
Ancyromonas sigmoides	AF174363	ATCC50267, Hudson Canyon box core sediment sample, Hudson Canyon, Atlantic Ocean
Ancyromonas sigmoides	AF053088	ATCC50267, Hudson Canyon box core sediment sample, Hudson Canyon, Atlantic Ocean
Caecitellus parvulus	HFCC300	Sediment, 19° 17.4′ S, 3° 52.2′ E, Angola Basin, South Atlantic Ocean (5424 m)
Caecitellus parvulus	HFCC301	Sediment, 19° 06.9′ S, 3° 52.0′ E, Angola Basin, South Atlantic Ocean (5423 m)
Caecitellus parvulus	AF174367	9° N East walls mussels bed, East Pacific Rise, Pacific Ocean (2500 m)
Caecitellus parvulus	AF174368	New Bedford Harbour, Massachusetts, USA (3 m)
Cafeteria roenbergensis	HFCC32	Brackish water, Baltic Sea near Hiddensee, Germany (surface water)
Cafeteria roenbergensis	HFCC33	Sediment, 16° 23.1′ S, 5° 27.0′ E, Angola Basin, South Atlantic Ocean (5388 m)
Cafeteria roenbergensis	HFCC34	17° 04.9′ S, 4° 40.8′ E, Angola Basin, South Atlantic Ocean (1 m)
Cafeteria roenbergensis	L27633	Trondheim Fjord, Norway (3 m)
Cafeteria roenbergensis	AF174364	9° N vent water – H_2S reactors, East Pacific Rise (2500 m)
Cafeteria sp.	AF174365	9° N vent water – H_2S reactors, East Pacific Rise (2500 m)
Cafeteria sp.	AF174366	Surface, Massachusetts, USA
Rhynchomonas nasuta	HFCC18	19° 06.9′ S, 3° 52.0′ E, Angola Basin, South Atlantic Ocean (1 m)
Rhynchomonas nasuta	HFCC302	Sediment, 19° 19.8′ S, 3° 55.6′ E, Angola Basin, South Atlantic Ocean (5425 m)
Rhynchomonas nasuta	HFCC303	Sediment, 18° 19.5′ S, 4° 43.0′ E, Angola Basin, South Atlantic Ocean (5414 m)
Rhynchomonas nasuta	HFCC99	Sediment, 79° 04.26′ N, 4° 09.12′ E, North Atlantic Ocean (2414 m)
Rhynchomonas nasuta	AF174377	9° N Chesapeake Bay, East Pacific Rise, Pacific Ocean (1 m)
Rhynchomonas nasuta	AF174378	9° N Biovent serpulid zone, East Pacific Rise, Pacific Ocean (2500 m)

sampling procedure), and displayed negative results in all cases (n = 10). On deck, sterile plastic syringes were used to fill organisms into sterile 50 ml tissue-culture flasks (Sarstedt) and diluted 1:3 with autoclaved artificial seawater (28.15 g NaCl, 0.67 g KCl, 5.51 g $MgCl_2 6H_2O$, 1.45 g $CaCl_2 2H_2O$, 6.92 g $MgSO_4$ $7H_2O$, 0.1 g KNO_3, 0.01 g $K_2HPO_4 3H_2O$ l^{-1}). In the laboratory, clonal cultures were established under sterile conditions both from surface water and from sediments using the serial dilution method and kept in culture at 10°C in artificial seawater with sterilised wheat grains at 1 atm. Experiments with deep-sea protists indicated that several organisms can be cultivated under normal atmospheric pressure (Patterson et al. 1993, Atkins et al. 2000, Arndt et al. 2003). Additional strains of *Ancyromonas sigmoides* (HFCC60) and *Cafeteria roenbergensis* (HFCC32) were isolated from shallow waters in the Southern Baltic Sea near Kloster (Hiddensee Island, Germany). We isolated 2 additional deep-sea strains from the North Atlantic (*Rhynchomonas nasuta* HFCC99 and *A. sigmoides* HFCC104) from samples collected in a way similar to that described above. Isolated cells were identified to species level using light microscopy following published descriptions of the respective species. All strains sequenced in this study are referred to by their accession numbers in the 'Heterotrophic Flagellate Culture Collection Cologne' (HFCC); all sequences retrieved from GenBank are referred to by their GenBank accession numbers.

DNA isolation and sequencing. The cultured isolates were grown to high densities (10^4 to 10^6 cells

ml^{-1}) and harvested by centrifugation. Collected cells were lysed and their DNA was isolated by a modified Kavenoff-Zimm procedure (Kavenoff & Zimm 1973, Steinbrück & Schlegel 1983). The entire SSU rDNA gene was amplified by PCR using general eukaryotic specific SSU rDNA primers (Table 2). Typical 50 µl PCR reaction conditions comprised 0.1 µM of each primer, 200 µM dNTPs, up to 100 ng genomic DNA, 1.5 mM $MgCl_2$, 1× reaction buffer and 1 U Ampli*Taq* DNA polymerase (Applied Biosystems). PCR was started with an initial denaturation step at 97°C for 5 min, after which the polymerase was added, followed by 35 cycles of 94°C for 30 s, 56°C for 45 s and 72°C for 2 min. PCR products were purified with the Rapid PCR Purification System of Marligen Biosciences (BIOCAT). Cycle sequencing was carried out with the BigDye Terminator Cycle Sequencing Kit Version 3.1 of Applied Biosystems. Cycle sequencing reactions were purified with AutoSeq G-50 columns from Amersham Biosciences and sequenced on an ABI 3100 automated sequencer. All these steps followed the manufacturers' protocols.

Table 2. External and internal SSU rDNA primers used in this study

Primer	5′-sequence-3′
18Sfor-Bodo[a]	CTG GTT GAT TCT GCC AGT AGT
18Srev-Bodo[a]	TGA TCC AGC TGC AGG TTC ACC
Kin-500for[b]	GAT TCC GGA GAG GGA GCC
Kin-500rev[b]	CTC TCC GGA ATC GAA CCC
Kin-740for[b]	TGT TAA AGG GTT CGT AGT TG
Kin-740rev[b]	TCA ACT ACG AAC CCT TTA AC
Kin-1220for[b]	GAC GAA CTA CAG CGA AGG C
Kin-1240rev[b]	GCC TTC GCT GTA GTT CGT C
Kin-1700for[b]	TGG TCG GTG GAG TGA TTT G
Kin-1720rev[b]	AAC AAA TCA CTC CAC CGA C
18Sfor[c,d]	AAC CTG GTT GAT CCT GCC AGT
18Srev[c]	TGA TCC TTC CGC AGG TTC ACC TAC
18Sfor-590[e]	CGG TAA TTC CAG CTC CAA TAG C
18Srev-600[e]	GCT ATT GGA GCT GGA ATT ACC G
18Sfor-900i[e]	ATT AAT AGG GAC AGT TGG GGG
18Sfor-1280[e]	TGC ATG GCC GTT CTT AGT TGG TG
18Srev-1300[e]	CAC CAA CTA AGA ACG GCC ATG C
400for[d]	AGA ATT AGG GTT CGA TTC CGG
450rev[d]	TAT TTC TTG TCA CTA CCT CCC
900for[d]	ATT AAT AGG GAC AGT TGG GGG
1000rev[d]	GAT TAA TGA AAA CAT CCT TGG
1350for[d]	ATT CCG ATA ACG AAC GAG ACC
1450rev[d]	ATC ACA GAC CTG TTA TTG CC

[a]External primers used for *Rhynchomonas nasuta*
[b]Internal primers used for *R. nasuta*
[c]External primers used for *Ancyromonas sigmoides*, *Amastigomonas debruynei*, *Cafeteria roenbergensis* and *Caecitellus parvulus*
[d]Internal primers used for *C. roenbergensis* and *C. parvulus*
[e]Internal primers used for *A. sigmoides* and *A. debruynei*

Phylogenetic analysis. Determined sequence fragments were assembled manually and aligned together with other sequences retrieved from GenBank/EMBL using the ClustalX multiple alignment program (Thompson et al. 1994). Uncorrected genetic distances (*p* distances) were calculated using the program PAUP Version 4.0b (Swofford 2000). Phylogenetic analyses were carried out by the distance matrix (neighbour-joining, NJ) method (Saitou & Nei 1987), the maximum parsimony (MP) method (Swofford & Olsen 1990) and the maximum likelihood (ML) method (Felsenstein 1981). The HKY85 (Hasegawa et al. 1985) model of nucleotide substitution was chosen for the NJ and ML analyses. The reliability of internal branches was assessed by bootstrapping (Felsenstein 1985) with 100 resamplings.

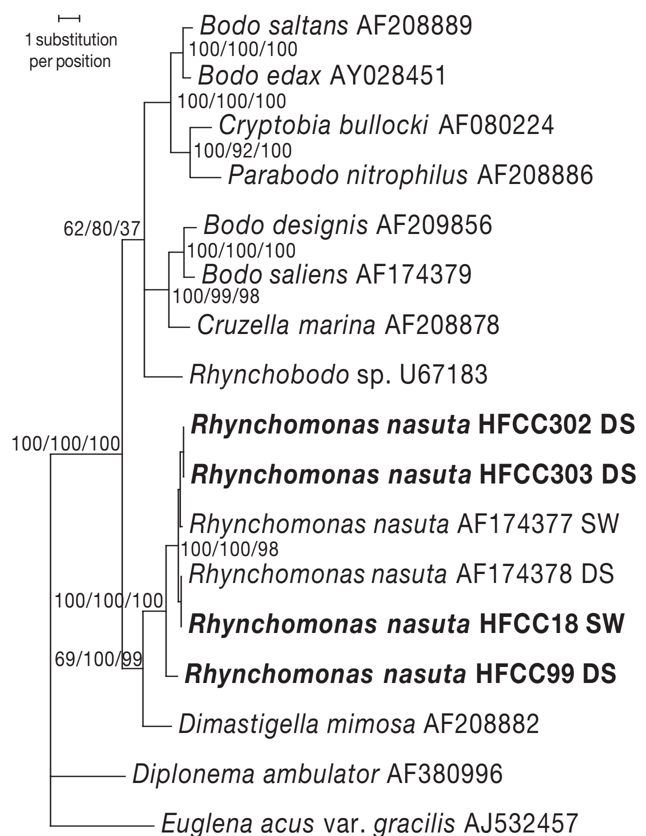

Fig. 1. Phylogenetic tree of Bodonidae using NJ (neighbour-joining), MP (maximum parsimony) and ML (maximum likelihood) methods. Numbers on the left are support values for the NJ tree, those in the middle for the ML tree, and those on the right for the MP tree (i.e. NJ/ML/MP). Tree rooted using *Diplonema ambulator* and *Euglena acus* var. *gracilis* as outgroups. Organisms sequenced in this study are in **bold**, with accession number for Heterotrophic Flagellate Culture Collection Cologne (HFCC); sequences retrieved from GenBank have GenBank accession number. DS: strains isolated from deep sea; SW: strains isolated from surface water. Number of informative sites for ML and MP analyses was 1050 and 787, respectively

RESULTS

Analysis of the complete SSU rDNA sequences conducted by NJ and ML applying the HKY85 model (Hasegawa et al. 1985) and by MP yielded principally identical phylogenetic trees. In the phylogenetic tree of Bodonidae (Fig. 1), all strains of *Rhynchomonas nasuta* formed a very well supported monophyletic clade. The branching order of most strains of *R. nasuta* was not well resolved. Only Strain HFCC99 branched off just below the cluster comprising all other isolates of *R. nasuta*, indicating a close relationship of the different strains of *R. nasuta*. Besides the 2 deep-sea strains from the South Atlantic (HFCC302 and HFCC303) that were sequenced in this study, 2 other sequences (GenBank) that had been isolated from different geographical locations (South Atlantic and Pacific) and habitats (surface water and hydrothermal vents) were nearly identical (Strains HFCC18 and AF174378; Table 3). As indicated by the branching order (Fig. 1), strain HFCC99 was genetically highly distant from other strains of *R. nasuta*, with distances ranging from 5.95 to 6.30%. Strain HFCC99 had an intron of 478 bases in a highly conserved region of the SSU, starting at Position 453. This region was excluded from the phylogenetic studies and did not occur in any other strain of *R. nasuta*. All other distances calculated were relatively low (1.5 to 1.8%).

All strains of *Amastigomonas debruynei* formed a clade with high bootstrap support (Fig. 2). According to the genetic distances of SSU rDNA, all 3 strains of *A. debruynei* (from deep sea and surface water of the South Atlantic, and the deep sea of the Sargasso Sea) were nearly identical, with a maximum genetic distance of 0.12% (Table 4). The isolates of *Ancyromonas sigmoides* formed a monophylum divided into 2 sister groups, one composed of both sequences retrieved from GenBank, the other of the 4 sequences obtained in this study. The 2 deep-sea strains of *A. sigmoides* from the South Atlantic (HFCC62 and HFCC63) were identical (Table 5), as had been shown for *R. nasuta*. Pairs of sequences retrieved from GenBank that had been isolated from different geographical regions (North Atlantic deep sea and Baltic Sea) were also nearly identical. The genetic distance between both closely related pairs of sequences obtained in this study was relatively low (1.98%) compared to the distance between our 4 sequences and both sequences retrieved from GenBank (9.41 to 10.05%).

Like the other species considered so far, all sequences of *Cafeteria roenbergensis* (and *Cafeteria* sp.) formed a monophyletic clade with high bootstrap sup-

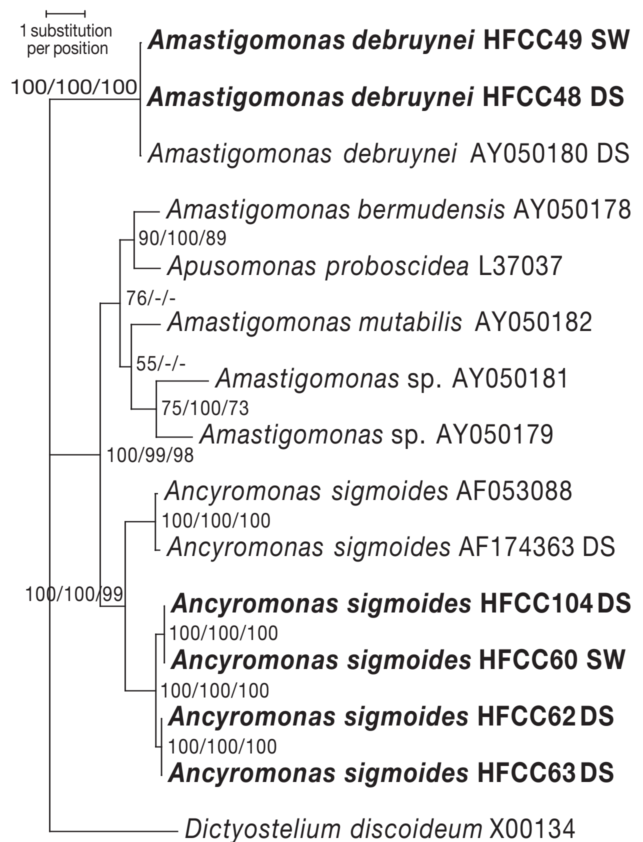

Fig. 2. Phylogenetic tree of Apusozoa using NJ, MP and ML methods (NJ/ML/MP). Number of informative sites for maximum likelihood and maximum parsimony analyses was 597 and 513, respectively. Further details as in Fig. 1

Table 3. *Rhynchomonas nasuta*. Uncorrected genetic distances (*p* distances) (%) of pairwise sequence comparison. Strains sequenced in this study are in **bold** and represented by accession number of Heterotrophic Flagellate Culture Collection Cologne (HFCC), and sequences retrieved from GenBank are represented by GenBank accession numbers (see Table 1 for collection details). DS: strains isolated from deep sea; SW: strains isolated from surface water

	(1)	**(2)**	(3)	**(4)**	(5)	**(6)**
(1) HFCC302 (DS)	0.00					
(2) HFCC303 (DS)	0.00	0.00				
(3) AF174378 (DS)	1.75	1.75	0.00			
(4) HFCC18 (SW)	1.80	1.80	0.05	0.00		
(5) AF174377 (SW)	1.30	1.30	1.50	1.55	0.00	
(6) HFCC99 (DS)	6.30	6.30	6.15	6.20	5.95	0.00

Table 4. *Amastigomonas debruynei*. Uncorrected *p* distances (%) of pairwise sequence comparison. Notation as in Table 3

	(1)	(2)	**(3)**
(1) HFCC49 (SW)	0.00		
(2) AY050180 (DS)	0.12	0.00	
(3) HFCC48 (DS)	0.06	0.06	0.00

Table 5. *Ancyromonas sigmoides*. Uncorrected *p* distances (%) of pairwise sequence comparison. Notation as in Table 3

	(1)	**(2)**	**(3)**	**(4)**	(5)	(6)
(1) HFCC62 (DS)	0.00					
(2) HFCC63 (DS)	0.00	0.00				
(3) HFCC104 (DS)	0.98	1.98	0.00			
(4) HFCC60 (SW)	1.98	1.98	0.00	0.00		
(5) AF053088	9.41	9.41	9.82	9.82	0.00	
(6) AF174363 (DS)	9.65	9.65	10.05	10.05	0.64	0.00

Table 6. *Cafeteria* spp. Uncorrected *p* distances (%) of pairwise sequence comparison. Notation as in Table 3

	(1)	**(2)**	(3)	**(4)**	**(5)**	(6)	(7)
(1) AF174365 (DS)	0.00						
(2) HFCC32 (SW)	0.00	0.00					
(3) AF174364 (DS)	0.00	0.00	0.00				
(4) HFCC34 (SW)	0.06	0.06	0.06	0.00			
(5) HFCC33 (DS)	0.31	0.31	0.31	0.25	0.00		
(6) L27633 (SW)	0.06	0.06	0.06	0.13	0.38	0.00	
(7) AF174366 (SW)	2.44	2.44	2.44	2.50	2.63	2.38	0.00

port (Fig. 3). In contrast to *Cafeteria* sp. sequence AF174365, *Cafeteria* sp. AF174366 not only branched below the cluster comprising all other sequences of *Cafeteria* with high bootstrap support, but was also genetically highly distant from the other sequences, with a maximum of 2.63% (Table 6). As within the other species considered, identical or nearly identical sequences were found over great geographic distances and within very different habitats from the Pacific, the South Atlantic, North America and the Baltic Sea.

Both pairs of sequences of *Caecitellus parvulus* were separated by very high genetic distances (11.73%, Table 7) as has been found within *Ancyromonas sigmoides*. As determined for *Rhynchomonas nasuta* and *Ancyromonas sigmoides*, both strains isolated from the South Atlantic deep sea were identical.

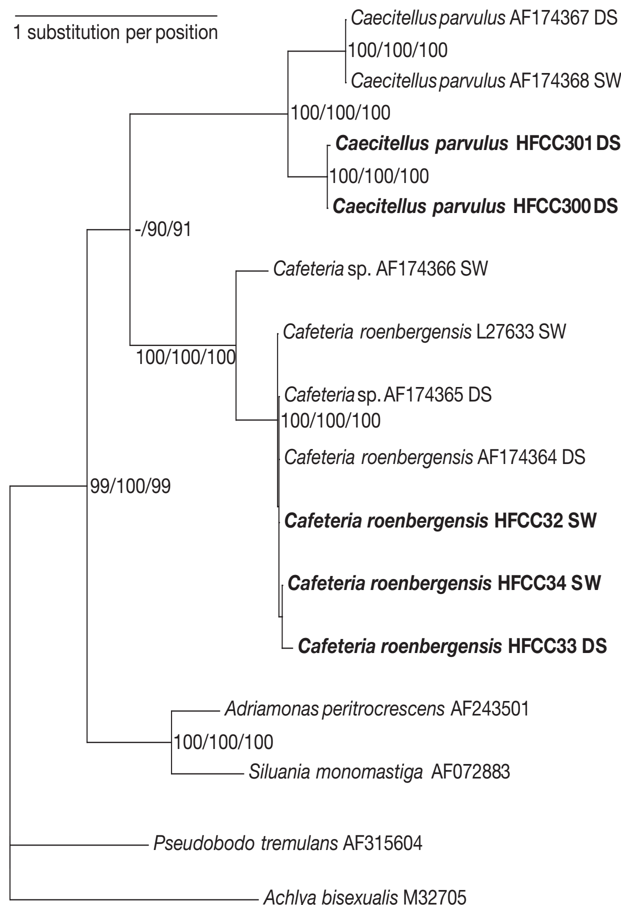

Fig. 3. Phylogenetic tree of Bicosoecida using NJ, MP and ML methods (NJ/ML/MP); only branching order of MP tree is shown. Number of informative sites for maximum likelihood and maximum parsimony analysis was 496 and 350. Further details as in Fig. 1

DISCUSSION

This study has shown that an exchange between flagellate populations between the upper parts of the water column and the deep sea may occur. At least some widely distributed flagellates such as *Amastigomonas debruynei* or *Cafeteria roenbergensis*, and some genotypes of other cosmopolitan heterotrophic flagellated morphospecies (*Rhynchomonas nasuta*, *Ancyromonas sigmoides* and *Caecitellus parvulus*) seem to have no particular biogeography, and thus appear to be cosmopolitan. In addition, the results for *A. sigmoides* and *C. parvulus* in particular, but also those for *R. nasuta*, indicate that world-wide species diversity may be greatly underestimated by the morphospecies concept. Genetic variation within some morphospecies was surprisingly high. The sequence dissimilarities recorded—up to 6.3% (*R. nasuta* HFCC99) or even 10.1% (*A. sigmoides* AF174363) dissimilarity from other strains of the relevant morphospecies—seem very unlikely within one and the same species. Although generally ignored so far, sibling species have previously been reported for the ciliate *Tetrahymena*

Table 7. *Caecitellus* spp. Uncorrected *p* distances (%) of pairwise sequence comparison. Notation as in Table 3

	(1)	(2)	**(3)**	**(4)**
(1) AF174368 (SW)	0.00			
(2) AF174367 (DS)	0.00	0.00		
(3) HFCC300 (DS)	11.73	11.73	0.00	
(4) HFCC301 (DS)	11.73	11.73	0.12	0.00

pyriformis (Nanney et al. 1998), coccoid green algae (Saéz et al. 2003) and heterotrophic flagellates (Von der Heyden et al. 2004), but our finding that 3 out of 5 species studied comprise several sibling species was unexpected.

All 5 species studied also contain clades of at least 2 (*Caecitellus parvulus*; Table 7) and up to 5 (*Rhynchomonas nasuta*; Table 3) similar strains. Genetic variation is comparatively low within these clades, with a maximum sequence divergence of 2.0% (*Ancyromonas sigmoides* HFCC60 and HFCC104). Even if the genetic variation within some of these clusters (*R. nasuta*, *C. parvulus* and *A. sigmoides*) may still seem too high for strains belonging to the same species, it is consistent with genetic variation found (e.g.) in the amoeboid species *Vannella simplex*, with maximum distances of 3.0% (Smirnov et al. 2002). The sequence dissimilarity of 1.5% reported for the species *R. nasuta* has previously led to the suggestion that both GenBank strains of *R. nasuta* should be separated into different species (Callahan et al. 2002). The only exceptions to variation in SSU rDNA genes are the morphospecies *Amastigomonas debruynei* and *Cafeteria roenbergensis*, whose morphology adequately defines species boundaries, at least for the presently available sequence pool. One must bear in mind that our analysis of SSU rDNA genes aimed only at determining genotype variability of morphospecies, and that our estimates of diversity may well constitute a lower estimate, with additional variability possibly being present within other genes. Moreover, in contrast to cloning of PCR products, direct sequencing of PCR products may not reveal potential intraspecific SSU rDNA variability, and may therefore fail to reveal other genotypes that are possibly present (Pecher et al. 2004).

The high genetic divergence between the 2 previously sequenced strains of *Caecitellus parvulus* and both strains of *C. parvulus* (maximum sequence dissimilarity of 11.7%) sequenced in this study are similar to distances between *C. parvulus* sequences retrieved from GenBank and the sequence of *Pseudobodo tremulans* (sequence divergence of 10.2%, Fig. 1). These distances are also similar for different genera of the Apusozoa (e.g. sequence dissimilarity between *Ancyromonas sigmoides* Strain AF053088 and *Apusomonas proboscidea* Strain L37037 is 12.3%, Fig. 2). Bearing in mind that a divergence of 6.1% between different strains of *Bodo saltans* was enough to suggest that this morphospecies should be divided into 2 new species (Callahan et al. 2002), it is difficult to envisage that the different strains of *A. sigmoides* and *C. parvulus* belong to one and the same species or even genus. Besides the need for ultrastructural studies to supplement and clarify molecular data, determination of the ecotype of the respective strains is of substantial interest (Finlay 2004). Were there are no differences in the ecotype, it could be argued that identification of cryptic species that is only possible by molecular methods would be useless since most taxonomic work is still done using light microscopy (Van der Strate et al. 2002). However, if ecotype differences exist, then not only the different ecotypes and thus genotypes must be identified, but the species complexes must be divided into either new species, or at least subspecies, as suggested for prokaryotes by Cohan (2002). Such division was recently necessary for the foraminiferan *Orbulina universa* (De Vargas et al. 1999). Current work (P. Selchow et al. unpubl. data) on 2 additional strains of *C. parvulus* from the Angola Basin with high genetic similarity to both strains sequenced in this study indicates that at least ultrastructural differences are present with regard to the species described by O'Kelly & Nerad (1998).

All 5 species we studied are described as bacterivorous benthic species and reported to belong to the 20 most abundant species of heterotrophic flagellates with world-wide distribution (Patterson & Lee 2000). Being poor swimmers, their common occurrence in marine pelagic environments points to the existence of specialised micro-environments based on detrital aggregates (e.g. Caron et al. 1982, Caron 1991). A diverse assemblage of heterotrophic flagellates lives in association with this marine detritus. Among these aggregate-associated flagellates, suspension- and raptorial-feeding species (such as the genera *Amastigomonas* and *Cafeteria*) are attached to or move about the surface of aggregates (Patterson et al. 1993). Since a significant proportion of detritus (and its associated microbial community) may leave the upper layers of the ocean and reach the deep-sea floor (Thiel et al. 1990, Gooday & Rathburn 1999) these aggregates are presumed to contribute mainly to the existence of active microbial communities in the deep-sea (Patterson et al. 1993, Atkins et al. 1998, Arndt et al. 2003). This could contribute to the wide geographical distribution of some species of flagellates (Caron 1991), as gene flow between the upper layers of the ocean and the deep sea is possible via sedimentation or water currents, as indicated by morphological (Arndt et al. 2003) and molecular (Atkins et al. 2000) studies. The occurrence of identical strains of species in different locations shows that genetic mixing across geographic barriers can occur (Atkins et al. 2000, this study). Some small organisms with high population sizes can be found wherever their required habitats are present, perhaps because of their ability to tolerate a wide range of environmental conditions (Finlay 2002, 2004). In contrast, some protistan morphospecies show a clear pattern of geographic distribution (Medlin et al. 2000, Coleman 2001) that cannot be resolved by SSU rDNA

sequences but only by more variable regions such as internal transcribed spacers (ITS).

Only a few molecular studies have investigated eukaryotic diversity in the deep sea. All of these have revealed an astonishing diversity of microbial eukaryotes, with many previously unknown taxa or even lineages (e.g. Díez et al. 2001, López-García et al. 2001), but only a few of these sequences could be determined to higher than genus level. It appears to be increasingly certain that protists, especially small protists of picoplanktonic size, form an important part of marine ecosystems, even in the deep sea. Their ecological role in nutrient recycling in these habitats is therefore much more important than currently recognised (Moreira & López-García 2002). If the different genotypes recognised within each morphospecies in the present study could be assigned to distinct ecotypes or to distinct ultrastructural morphotypes, then at least the species *Rhynchomonas nasuta*, *Ancyromonas sigmoides* and *Caecitellus parvulus* will be unsustainable as morphospecies and the global species richness of heterotrophic flagellates must be considered highly underestimated, as previously suggested with regard to protists in general (Foissner 1999).

Our study underlines the need for the use of molecular techniques based on rDNA sequencing, which could lead to significant changes in flagellate taxonomy (Aron et al. 2004).

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