Molekularbiologische Untersuchungen zur Biodiversität heterotropher Flagellaten

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Einleitung

Protisten sind eine heterogene Zusammenfassung mikrobieller Eukaryoten bestehend aus Organismen wie Protozoa, einzelligen Algen oder Schleimpilzen (Hausmann et al. 2003). Sie entwickelten sich vor etwa 1.5 bis 2 Milliarden Jahren durch Endosymbiose aus Prokaryoten (Margulis et al. 1993). Die meisten Protisten sind mikroskopisch klein (< 100 µm) und spielen eine entscheidende Rolle in natürlichen Lebensräumen (Corliss 2002). Frei-lebende Protozoa sind phagotroph und kontrollieren die Abundanz von Bakterien und anderen Mikroben, einzellige Algen sind für die Fixierung des größten Teils an CO_2 in aquatischen Lebensräumen verantwortlich, und Schleimpilze sind wichtige Konsumenten von Bakterien und Pilzen im Boden, um nur einige Beispiele für ihre entscheidende Rolle in natürlichen Lebensräumen zu nennen. Neben Bakterien und kleinen Metazoa sind Protisten die wichtigste Komponente im mikrobiellen Nahrungsgewebe aquatischer Ökosysteme (Arndt et al. 2000). Nach Viren und Prokaryoten stellen Protisten die individuenreichste Organismengruppe überhaupt dar, mit Abundanzen von 10^4 bis 10^7 Individuen $\cdot$ m^{-2}. Als Gruppe weisen Protisten eine unglaubliche morphologische und ultrastrukturelle Vielfalt auf und ihre Morphologie – und somit das morphologische Artkonzept – ist seit über 200 Jahren der beherrschende Faktor für ihre Bestimmung (Hausmann et al. 2003). Bis heute ist das Maß ihrer Artenvielfalt nicht bekannt und es besteht kein Konsens ihre Phylogenie betreffend (Andersen 1998).

Flagellaten dominieren Protistengemeinschaften in planktischen Lebensräumen. Sie umfassen eine taxonomisch sehr diverse Gruppe einzelliger oder koloniebildender Organismen (oder einen Lebensabschnitt anderer Protisten), welche sich durch das Vorhandensein eines oder mehrerer Flagellen, bzw. Cilien, in der trophischen Phase auszeichnen und keinen Makronukleus besitzen. Sie stellen eine rein morphologisch definierte, polyphyletische Gruppe eukaryotischer Einzeller dar, welche in 111 Ordnungen, 60 Klassen, 16 Stämmen und 3 Königreichen eingeordnet werden und wahrscheinlich die ursprünglichste eukaryotische Organisationsform sind (Cavalier-Smith 2000). Sie besitzen meistens eine begrenzte Anzahl morphologischer Merkmale, was ihre Identifizierung erschwert und oftmals elektronenmikroskopische Aufnahmen nötig macht. Primäre Bestimmungsmerkmale sind, neben ihrer Färbung, die Anordnung und der Aufbau ihrer Flagellen, ebenso wie die allgemeine Körperform und Bedeckung. Auf unteren taxonomischen Ebenen gleichen sich die morphologischen Merkmale häufig und komplizierte, polymorphe Lebenszyklen einiger Flagellaten erschweren ihre Bestimmung zusätzlich (Medlin et al. 2000).

In der Ökologie gibt es eine Reihe grundlegender Muster (Lawton 1999). Eines dieser makroökologischen Muster ist das der beinahe schon kanonischen log-normalen Artenvielfalt. Hutchinson und MacArthur (1959) hatten beobachtet, dass die Anzahl an Habitaten und demzufolge an Arten (S), umgekehrt proportional zur Körperlänge (L) im Quadrat abnahm ($S = L^{-2}$; Abbildung I). Eine ähnliche Beziehung wurde von Siemann et al. (1996) für die Beziehung zwischen Artenzahl (S) und Abundanz (I) beobachtet ($S = I^2$). Hiervon abweichende Muster wurden von Robert M. May (1988) für terrestrische und von Tom Fenchel (1993) für aquatische Gemeinschaften gefunden. Robert

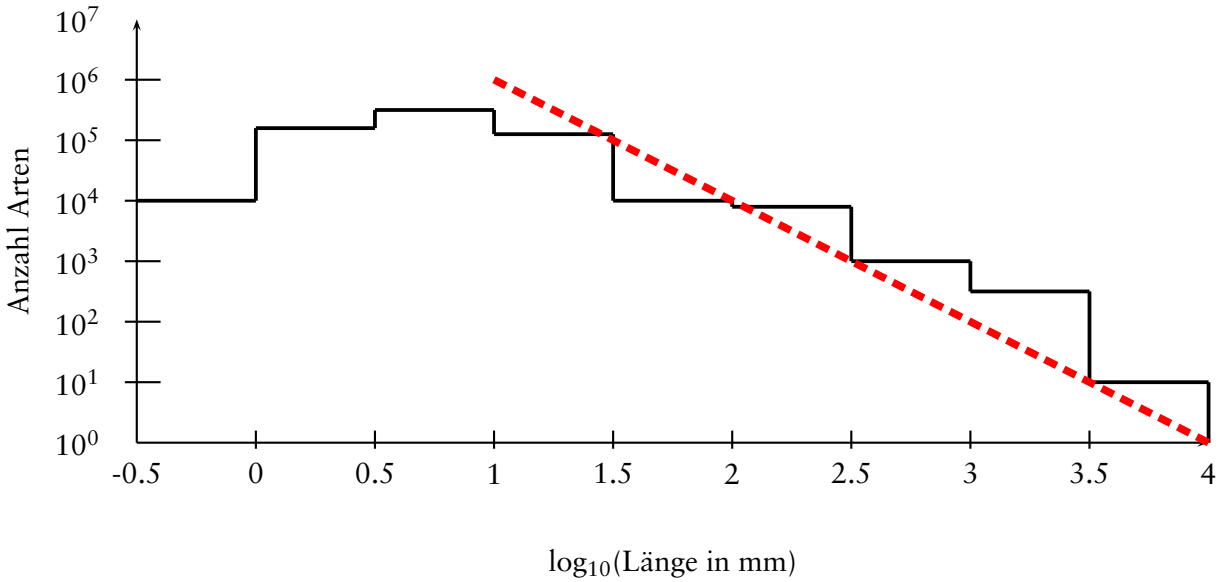

Abbildung I: Schätzung der Anzahl Arten pro Größenklasse (schwarze Linie) und des Verhältnisses $S = L^{-2}$ (S Anzahl Arten, L Länge; rote Linie). Quelle: Robert M. May „How many species are there on earth?“ Science 241:1441-1449, 1988.

M. May (1988) hatte die Anzahl beschriebener Arten in den jeweiligen Größenklassen zusammengetragen. Dabei nehmen die Artenzahlen in logarithmischen Größenklassen mit abnehmender Größe zu mit der a priori Erwartung, dass es insgesamt mehr kleine, als große Arten geben solle. Bei einem Schwellenwert, welcher annähernd bei einer Größe von 1 mm liegt, nehmen die Artenzahlen hingegen wieder kontinuierlich ab (Abbildung I). Mit Ausnahme einiger weniger Taxa liegen alle Protistenarten unterhalb dieses Schwellenwertes. Extrapoliert man die von Robert M. May dargelegte Beziehung, müssten bereits in der Größenklasse von 1 mm 10 bis 50 Millionen Arten vorhanden sein; die Anzahl an Protozoa müsste demzufolge astronomisch hoch sein und bei über 10^8 Arten liegen. Wie Robert M. May bemerkte, ist dem aber augenscheinlich nicht so (Tabelle I). Weltweit sind etwa 720000 Insektenarten beschrieben (May 1997) und die geschätzte Gesamtzahl mag bei über 5 Millionen liegen (Gaston 1992). Demgegenüber sind aber gerade einmal 30000 Protozoa (Fenchel 1993) beschrieben und Schätzungen der Gesamtartenzahl kommen auf ungefähr 100000 (Corliss 1982, May 1997) bis 200000 („Global Biodiversity Estimate for the UN“, Heywood 1995). Selbst die am besten untersuchten Protisten – die Ciliaten – sind mit geschätzten 3000 Arten vergleichsweise artenarm (Finlay & Fenchel 1999), auch wenn anerkannte α-Taxonomen auf diesem Gebiet, wie Wilhelm Foissner (1999), ihre Gesamtzahl um den Faktor 10 höher schätzen (40000 Arten). Die so wichtige Gruppe der Flagellaten brachte es 1982 (Corliss 1982) ohne Dinoflagellaten (4200 Arten) und Haptophyta (1500 Arten) auf 200 Arten. Schätzungen der Gesamtzahl heterotropher Flagellaten (ohne Dinoflagella-

Tabelle I: Anzahl benannter lebender Arten und Schätzung der Gesamtartenzahl.

Gruppe	Anzahl benannter Arten†	Geschätzte Gesamtartenzahl†
Protozoa	40	100
Algen	40	300
Pflanzen	270	320
Pilze	70	500
Tiere		
Wirbeltiere	45	50
Fadenwürmer	15	500
Weichtiere	70	120
Gliederfüßer insgesamt	855	4650
Krebse	40	150
Spinnentiere	75	500
Insekten	720	4000
Andere Tiere	95	250
Insgesamt	**1500**	**6800**

† Anzahl in Tausend.

Quelle: Robert M. May „The Dimension of Life on Earth“ in Nature and Human Society: The Quest for a Sustainable World, Peter H. Raven (Herausgeber), National Academy Press, Washington, D. C., 1997.

ten) kommen auf 3000 Arten (Lee & Patterson 1998). Das geschätzte „grand total“ an Eukaryoten mag bei 6.8 Millionen Arten liegen (May 1997). Protozoa würden demzufolge nur 1,47 % aller Arten weltweit stellen.

Eine Erklärung für diese geringen Artenzahlen bei Protisten liegt möglicherweise in ihren hohen Abundanzen, welche zusammen mit einer einfachen Verbreitungsmöglichkeit (Griffin et al. 2002) zu einem weltweiten Vorkommen aller Arten in ihrem jeweiligen Habitat führt („everything is (almost) everywhere, the environment selects“, Fenchel & Finlay 2003). Nach Ernst Mayr (1942) ist aber für die Entstehung neuer Arten eine räumliche und zeitliche Trennung von Populationen notwendig. Da diese Ansichtsweise in der Protistologie nach wie vor Lehrbuchcharakter hat, muss bei einer weltweiten Verbreitung von Protisten die Rate allopatrischer Artbildung niedrig sein und folglich wäre mit einer niedrigen Gesamtartenzahl zu rechnen (Finlay 2002). Prokaryoten, welche in der Mehrzahl noch eine Größenordnung unter den Protisten liegen, bei zugleich höheren Gesamtzahlen (10^{30} Individuen weltweit), müssten Autoren wie Bland J. Finlay und Tom Fenchel zufolge, entsprechend wenige Arten aufweisen, und in der Tat sind lediglich 4500 Arten benannt (Torsvik et al. 2002). Aktuelle Schätzungen ihrer Gesamtartenzahl gehen aber in die Millionen (Curtis et al. 2002, Torsvik et al. 2002), ja sogar Milliarden (Dykhuizen 1998). Hochrechnungen basierend auf der kleinen ribosomalen Untereinheit (SSU) kommen je nach Maß für die zulässige intraspezifische genetische Distanz auf 35000 (maximal 3 % genetische Distanz) bis 325000 (0 % genetische Distanz) Bakterienarten (Schloss & Handelsmann 2004).

Robert M. May (1988) und Wilhelm Foissner (1999) haben als Grund für die möglicherweise zu niedrige Artenzahl an Protisten fehlendes taxonomisches Interesse, und demzufolge fehlende taxonomische Arbeit auf diesem Gebiet, genannt. Demzufolge müssten noch eine Vielzahl Arten an nicht näher untersuchten Orten dieser Welt auf ihre Entdeckung warten (z. B. im Boden, im tropischen Regenwald oder im Tiefseebenthos). Aktuelle molekularbiologische Arbeiten konnten in der Tat eine Fülle unbekannter Phylotypen in natürlichen Lebensräumen entdecken und zeigen, dass die mikrobielle Diversität viel größer ist, als mit konventionellen Methoden aufgedeckt werden kann (Moon-van der Staay et al. 2001, López-García et al. 2001, Dawson & Pace 2002). Es scheint aber unwahrscheinlich, dass mehrere Größenordnungen an Arten, mit klassischen Methoden übersehen wurden (Lawton 1998). Des Weiteren könnten die Diversitätsmaxima in weniger gut untersuchten Gruppen, wie etwa den heterotrophen Flagellaten, liegen, aber auch dies scheint unwahrscheinlich (Lawton 1998).

Besondere Aufmerksamkeit muss grundsätzlichen Problemen mit dem morphologischem Artkonzept gewidmet werden (Schlegel & Meisterfeld 2003). Die unzureichende taxonomische Auflösung der morphologischen Merkmale vieler Protisten ist möglicherweise der entscheidende Grund für ihre geringen Artenzahlen (May 1988). So ist es z. B. nicht möglich einige marine, kokkenähnliche Picoplankter („brown tiny balls“) morphologisch über das taxonomische Level der Klasse zu bestimmen (Potter et al. 1997); einige morphologisch identische Stämme der Art *Tetrahymena pyriformis* können sich nicht miteinander paaren (Nanney et al. 1998); verschiedene Stämme der Morphoart *Neobodo designis* haben unterschiedliche Toleranzen gegenüber dem Salzgehalt ihrer Umgebung (Ekelund 2002); und grundsätzlich können morphologisch identische Stämme große genetische Unterschiede aufweisen (Scheckenbach et al. 2005). Das morphologische Artkonzept führt demnach möglicherweise zu einem „lumping“ unterschiedlicher Arten unter dem Mantel einer Morphoart (Patterson & Lee 2000). Aktuelle Arbeiten deuten auf ein eher hohes Maß an kryptischer Diversität hin und Bakteriologen, ebenso wie Hefe-Systematiker, sind sich sich seit längerem darüber im Klaren, dass eine morphologische Klassifikation eigentlich bedeutungslos ist (Lachance 2004). Sollte eine hohe Anzahl kryptischer Arten, d. h. Arten, welche mit dem derzeit vorherrschenden morphologischen Artkonzept nicht mehr unterschieden werden können („sibling species, cryptic species, genetic species, physiological/ecological species“), der Grund für die geringe Artenzahl an Protisten sein? Sollte John J. Cairns (1993) mit seiner Vermutung Recht behalten, dass Morphoarten lediglich eine Fassade sind, hinter welcher sich eine Fülle taxonomisch eigenständiger Einheiten verbergen?

Diese Arbeit soll am Beispiel heterotropher Flagellaten, Hinweise darauf liefern, dass die Diversität von Protisten durch das morphologische Artkonzept unterschätzt wird. Darüber hinaus soll versucht werden eine Abschätzung über das Maß an kryptischer Diversität zu geben. Hierzu soll der Grad an intraspezifischer genetischer Divergenz und somit möglicher kryptischer Diversität der ökologisch so bedeutsamen Gruppe heterotropher Flagellaten ermittelt werden. Dazu wurden einige der weltweit häufigsten Arten heterotropher Flagellaten untersucht (*Amastigomonas debruynei, Ancyromonas sigmoides, Apusomonas proboscidea, "Bodo" curvifilus, Bodo saltans, Cafeteria roenbergensis, Caecitellus parvulus, Dimastigella mimosa, Neobodo designis, Neobodo*

saliens, Parabodo caudatus, Pseudobodo tremulans, Procryptobia sorokini, Rhynchobodo sp. und *Rhynchomonas nasuta*). Eine Datenbankrecherche soll die kryptische Diversität in Gruppen, welche ausschließlich Protisten enthalten, ermitteln. Als Maß für die kryptische Diversität wird dabei die genetische Divergenz innerhalb einer Morphoart angenommen ebenso wie deren Phylogenie. Hierbei spielt es zunächst einmal keine Rolle, was der Grund für das Entstehen kryptischer Arten ist, da unterschiedliche Arten prinzipiell mit der Zeit genetisch divergieren und dies wiederum mit geeigneten molekularen Markern nachweisbar ist. Als molekularen Marker dient die kleine ribosomale Untereinheit (SSU), deren Auflösung auf der taxonomischen Ebene der Morphoart bei Protisten in aller Regel als ausreichend angesehen wird (Medlin et al. 2000, Avise 2004).

Da Artbildung in Sympatrie eine weitaus bedeutendere Rolle zu spielen scheint, als bisher angenommen (Tautz 2003), und dies am leichtesten entlang steiler ökologischer Gradienten (Dieckmann & Doebeli 1999, Doebeli & Dieckmann 2003), wurden Stämme von geographisch und ökologisch unterschiedlichen Orten isoliert, um evolutionär relevante Muster zu finden. Der Vergleich von Stämmen aus Süßwasser mit Stämmen aus marinen Habitaten und insbesondere der Tiefsee (May 1992, Morin & Fox 2004), schien besonders viel versprechend, um eventuell ökologische, bzw. biogeographische Muster, innerhalb von Morphoarten heterotropher Nanoflagellaten (Zellgröße der untersuchten Arten $\leqslant 16\ \mu m$), welche weltweit verbreitet sind und zu den ökologischen Generalisten zählen, zu finden. Die Arbeit gliedert sich in 4 Kapitel.

- Kapitel 1 untersucht die intraspezifische genetische Divergenz innerhalb von Morphoarten, für welche Stämme zugleich von der Oberfläche und der Tiefsee des Südatlantiks isoliert wurden, und geht der Frage nach, ob ein und dieselbe „Art“ in derart unterschiedlichen, geographisch getrennten Lebensräumen vorkommen kann.

- Kapitel 2 untersucht die intraspezifische genetische Divergenz innerhalb von Morphoarten, für welche Stämme aus unterschiedlichen Habitaten isoliert wurden, und geht insbesondere der Frage nach, inwieweit die ökologischen Unterschiede zwischen Süßwasser und marinen Habitaten für ein und dieselbe „Art“ ein Hindernis für ihre Verbreitung darstellen, bzw. inwieweit unterschiedliche Habitate unterschiedliche ökologische Nischen darstellen, und somit andere Arten beherbergen sollten.

- Kapitel 3 untersucht die intraspezifische genetische Divergenz und Ultrastruktur von *Caecitellus* spp., und geht der Frage nach, worin die Unterschiede zwischen genetisch divergierenden Stämmen einer kryptischen Morphoart liegen.

- Kapitel 4 versucht eine Abschätzung des Maßes an kryptischer Diversität anhand der von mir bearbeiteten Morphoarten, zusammen mit Daten anderer Protistenarten aus GenBANK, zu geben.

Kooperationspartner

Für die Auswertung in der vorliegenden Arbeit wurden teilweise Daten Dritter zur Verfügung gestellt, bzw. Daten mit Hilfe Dritter erhoben. Dies waren im einzelnen:

- Die morphologischen und v. a. ultrastrukturellen Untersuchungen von *Caecitellus paraparvulus* (DQ220712) und *Caecitellus pseudoparvulus* (DQ220713) wurden von Petra Selchow bei Prof. K. Hausmann, AG Protozoologie, Institut für Biologie/Zoologie, Freie Universität Berlin, durchgeführt. Von Petra Selchow stammen ebenfalls Teile von Kapitel 3.

- Die externen und internen Primer wurden von Claudia Wylezich erstellt.

- Im Rahmen der „Heterotrophic Flagellate Culture Collection Cologne“ (HFCC) standen die Stämme mit folgenden GenBANK-Zugriffsnummern zur Verfügung: AY827841–AY827846, AY827849–AY827852, AY827855, DQ207563, DQ207-567, DQ207569–DQ207571, DQ207576–DQ207581, DQ207589–DQ207593, DQ207595, DQ220712–DQ220713, DQ220718.

- *Apusomonas proboscidea* (DQ207568) wurde von Nina Loquay isoliert und sequenziert. Die Fragmente wurden von Rosita Bieg zusammengefügt und korrigiert.

- *Bodo saltans* (DQ207574) und *Rhynchomonas nasuta* (DQ207598) wurden von Markus Weitere isoliert, bzw. zur Verfügung gestellt.

- *Caecitellus pseudoparvulus* DQ230538 stammt aus der „American Type Culture Collection“ (*Caecitellus parvulus* ATCC50091).

Part I.

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

Abstract

Whereas much is known about the biodiversity of prokaryotes and macro-organisms in the deep sea, knowledge concerning the biodiversity of protists remains very limited. Molecular studies have changed our view of the marine environments and have revealed an astonishing number of previously unknown eukaryotic organisms. Morphological findings show 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, 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

Introduction

Heterotrophic flagellates are recognised as being fundamental components of aquatic ecosystems. Within planktonic and benthic food webs these micro-organisms function as nutrient remineralizers 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, Ekelund & Patterson 1997, Tong 1997a, 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 (Turkey et al. 1988, Turley & Carstens 1991, Atkins et al. 1998, Hausmann et al. 2002a, 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, Patterson & Lee 2000, Atkins et al. 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 abundances and the possibility to form 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 micro-organisms 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 from 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 ecolog-

ically 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 protist 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 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 *Caecitellus 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 the expedition with R/V "Meteor" (Cruise 48, leg 1; Expedition DIVA 1) in the oligotrophic South Atlantic, Angola Basin (a detailed overview is given in Table 1.1). They were collected from surface waters and from multicorer samples from depth between 5300 and 5500 m. Salinity was about 37 ‰ 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 used for sampling. The top and the bottom of corers 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 the transport through the water column was checked with "blind" samples (autoclaved material exposed with the sampling device during the whole 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 sea water (35 ‰: 28.15 g NaCl, 0.67 g KCl, 5.51 g $MgCl_2 \cdot 6H_2O$,

Table 1.1: Location and depth of collection of all species studied, with accession numbers for GenBank. Species sequenced in this study are in **bold**.

Species name	Accession No.	Sample location
Amastigomonas debruynei	AY827842	18°19.5'S 4°43.0'E, -2 m, Angola Basin, South Atlantic Ocean
Amastigomonas debruynei	AY827841	Sediment, 18°25.3'S 4°44.0'E, -5392 m, Angola Basin, South Atlantic Ocean
Amastigomonas debruynei	AY050180	Sargasso Sea, -2500 m, Atlantic Ocean
Ancyromonas sigmoides	AY827845	Sediment, 17°11.6'S 4°45.9'E, -5415 m, Angola Basin, South Atlantic Ocean
Ancyromonas sigmoides	AY827844	Sediment, 18°19.5'S 4°43.0'E, -5392 m, Angola Basin, South Atlantic Ocean
Ancyromonas sigmoides	AY827843	Brackish water, Baltic Sea near Hiddensee, Germany
Ancyromonas sigmoides	AY827846	79°07.27'N 4°07.95'E, -1804 m, North Atlantic Ocean
Ancyromonas sigmoides	AF174363	ATCC50267, box core sediment sample, Hudson Canyon, Atlantic Ocean
Ancyromonas sigmoides	AF053088	ATCC50267, box core sediment sample, Hudson Canyon, Atlantic Ocean
Caecitellus parvulus	AY827848	Sediment, 19°06.9'S 3°52.0'E, -5423 m, Angola Basin, South Atlantic Ocean
Caecitellus parvulus	AY827847	Sediment, 19°17.4'S 3°52.2'E, -5424 m, Angola Basin, South Atlantic Ocean
Caecitellus parvulus	AF174368	New Bedford Harbour, -3 m, Massachusetts, USA
Caecitellus parvulus	AF174367	9°N East walls mussels bed, -2500 m, East Pacific Rise, Pacific Ocean
Cafeteria roenbergensis	L27633	Trondheim Fjord, -3 m, Norway
Cafeteria roenbergensis	AY827851	17°04.9'S 4°40.8'E, -1 m, Angola Basin, South Atlantic Ocean
Cafeteria roenbergensis	AY827850	Sediment, 16°23.1'S 5°27.0'E -5388 m, Angola Basin, South Atlantic Ocean
Cafeteria roenbergensis	AY827849	Brackish water, Baltic Sea near Hiddensee, Germany
Cafeteria roenbergensis	AF174364	9°N vent water, H_2S reactors, -2500 m, East Pacific Rise
Rhynchomonas nasuta	AY827855	Sediment, 79°04.26'N 4°09.12'E, -2414 m, North Atlantic Ocean
Rhynchomonas nasuta	AY827854	Sediment, 18°19.5'S 4°43.0'E, -5414 m, Angola Basin, South Atlantic
Rhynchomonas nasuta	AY827853	Sediment, 19°19.8'S 3°55.6'E, -5425 m, Angola Basin, South Atlantic
Rhynchomonas nasuta	AY827852	19°06.9'S 3°52.0'E, -1 m, Angola Basin, South Atlantic
Rhynchomonas nasuta	AF174378	9°N Biovent serpulid zone, -2500 m, East Pacific Rise, Pacific Ocean
Rhynchomonas nasuta	AF174377	9°N Chesapeake Bay, -1 m, East Pacific Rise, Pacific Ocean

1.45 g $CaCl_2 2 \cdot H_2O$, 6.92 g $MgSO_4 \cdot 7H_2O$, 0.1 g KNO_3, 0.01 g $K_2HPO_4 \cdot 3H_2O \cdot l^{-1}$). 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* (AY827843) and *Cafeteria roenbergensis* (AY827849) were isolated from shallow waters in the Southern Baltic Sea near Kloster (Island Hiddensee, Germany). We isolated 2 additional deep-sea strains from the North Atlantic (*Rhynchomonas nasuta* AY827855 and *A. sigmoides* AY827846) from samples collected in a similar way as described above. Isolated cells were identified to the species level using light microscopy following descriptions of the respective species. All strains sequenced in this study and 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 (10^4–10^6 cells $\cdot$ ml^{-1}) and harvested by centrifugation. Collected cells were lysed and their DNA was isolated using 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 1.2). 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

Table 1.2: 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*

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 from Marligen Biosciences (BIOCAT). Cycle sequencing was carried out with the BigDye Terminator Cycle Sequencing Kit V 3.1 from 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 were performed following the manufacturer's protocols.

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 programme 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.

Results

Analysis of the complete SSU rDNA sequences conducted by NJ and ML methods applying the HKY85 model (Hasegawa et al. 1985), and by MP yielded principally identical phylogenetic trees. In the phylogenetic tree of Bodonidae (Fig. 1.1), 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 sequence AY827855 branched off just below the cluster comprising all other isolates of *R. nasuta*, indicated a close relationship of the different strains of *R. nasuta*. Besides the 2 deep-sea strains from the South Atlantic (AY827853 and AY827854) 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 (AY827852 and AF174378; Table 1.3). As indicated by the branching order (Fig. 1.1), sequence AY827855 was genetically highly distant from other strains of *R. nasuta*, with distances ranging from 5.95 up to 6.30 %. Sequence AY827855 had an intron of 478 bases in a highly conserved region of the SSU, starting at Position 453. This region was excluded from 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. 1.2). According to the genetic distances of SSU rDNA, all 3 strains of *A. debruynei* (from deep sea and surface water of South Atlantic, and the deep sea of Sargasso Sea)

Table 1.3: *Rhynchomonas nasuta*. Uncorrected genetic distances (*p*-distances) (%) of pairwise sequence comparison. Strains sequenced in this study are in **bold**. All sequences are referred to by their GenBank accession numbers (see Table 1.1 for collection details). DS: strains isolated from deep sea; SW: strains isolated from surface water.

	(1)	**(2)**	(3)	**(4)**	(5)	**(6)**
(1) AY827853 (DS)	0.00					
(2) AY827854 (DS)	0.00	0.00				
(3) AF174378	1.75	1.75	0.00			
(4) AY827852 (SW)	1.80	1.80	0.05	0.00		
(5) AF174377	1.30	1.30	1.50	1.55	0.00	
(6) AY827855 (DS)	6.30	6.30	6.15	6.20	5.95	0.00

were nearly identical, with a maximum genetic distance of 0.12 % (Table 1.4). 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 (AY827844 and AY827845) were identical (Table 1.5), 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 support (Fig. 1.3). 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 1.6). 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.

Table 1.4: *Amastigomonas debruynei*. Uncorrected *p*-distances (%) of pairwise sequence comparison. Notation as in Table 1.3.

	(1)	(2)	**(3)**
(1) AY827842 (SW)	0.00		
(2) AY050180	0.12	0.00	
(3) AY827841 (DS)	0.06	0.06	0.00

Table 1.5: *Ancyromonas sigmoides*. Uncorrected *p*-distances (%) of pairwise sequence comparison. Notation as in Table 1.3.

	(1)	(2)	(3)	(4)	(5)	(6)
(1) AY82744 (DS)	0.00					
(2) AY827845 (DS)	0.00	0.00				
(3) AY827846 (DS)	0.98	1.98	0.00			
(4) AY827843 (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 1.6: *Cafeteria* spp. Uncorrected *p*-distances (%) of pairwise sequence comparison. Notation as in Table 1.3.

	(1)	(2)	(3)	(4)	(5)	(6)	(7)
(1) AF174365 (DS)	0.00						
(2) AY827849 (SW)	0.00	0.00					
(3) AF174364 (DS)	0.00	0.00	0.00				
(4) AY827851 (SW)	0.06	0.06	0.06	0.00			
(5) AY827850 (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

Both pairs of sequences of the genus *Caecitellus* were separated by very high genetic distances (11.73 %, Table 1.7) as has been found within the genus *Ancyromonas sigmoides*. As determined for *Rhynchomonas nasuta* and *Ancyromonas sigmoides*, both strains isolated from the South Atlantic deep sea were identical.

Discussion

This study has shown that an exchange of 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 (*Rhyn-*

Table 1.7: *Caecitellus parvulus*. Uncorrected *p*-distances (%) of pairwise sequence comparison. Notation as in Table 1.3.

	(1)	(2)	(3)	(4)
(1) AF174368 (SW)	0.00			
(2) AF174367 (DS)	0.00	0.00		
(3) AY827847 (DS)	11.73	11.73	0.00	
(4) AY827848 (DS)	11.73	11.73	0.12	0.00

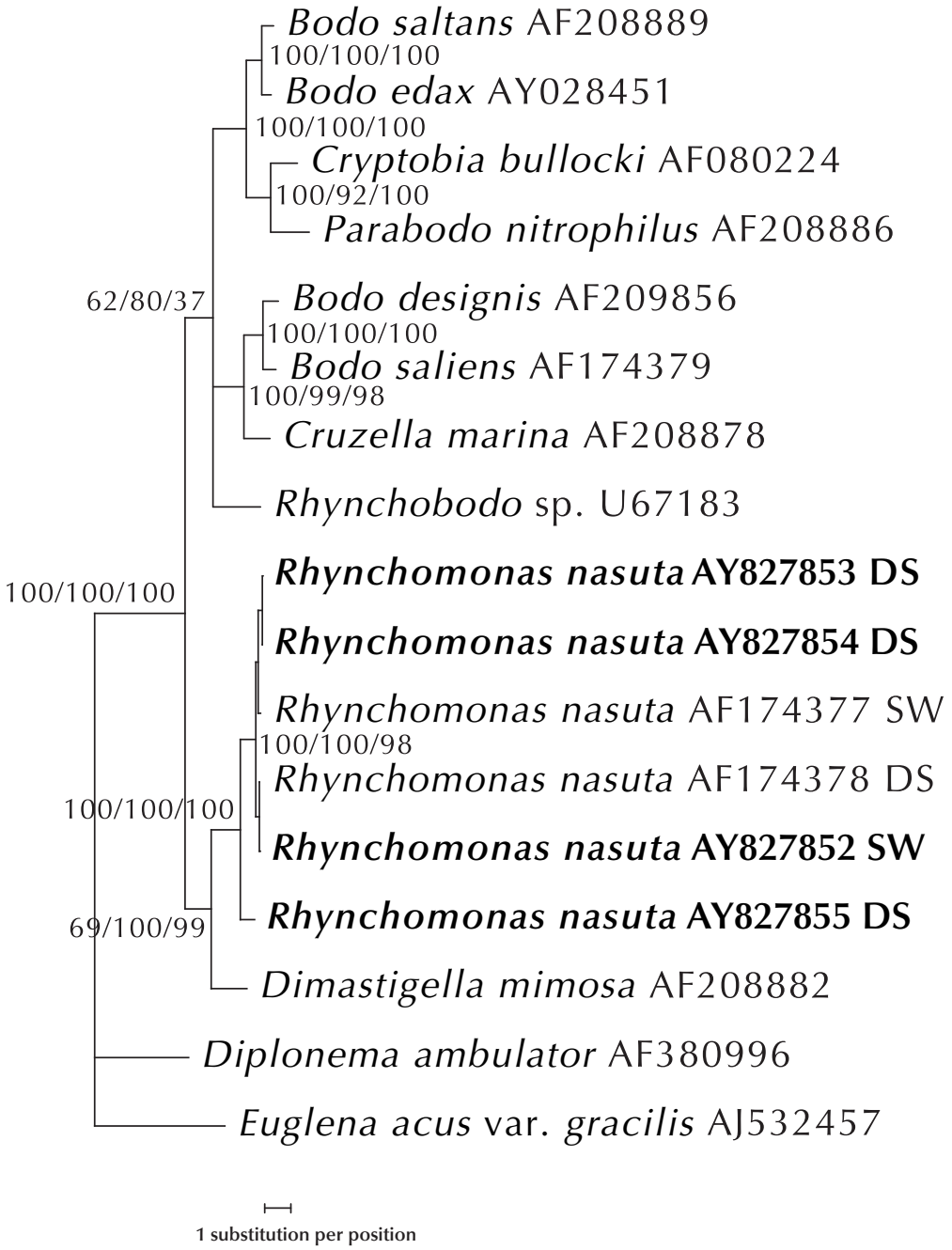

Figure 1.1: Phylogenetic tree of Bodonidæ using NJ (neighbour joining), MP (maximum parsimony) and ML (maximum likelihood) methods. Numbers on the left are support values for the NJ tree, those in the middle for the ML tree, and those on the right for the MP tree (i. e. NJ/ML/MP). Tree was rooted using *Diplonema ambulator* and *Euglena acus* var. *gracilis* as outgroups. Organisms sequenced in this study are in **bold**. All sequences are referred to by their GenBank accession numbers. DS: strains isolated from deep sea; SW: strains isolated from surface water. The number of informative sites for ML and MP analysis was 1050 and 787, respectively.

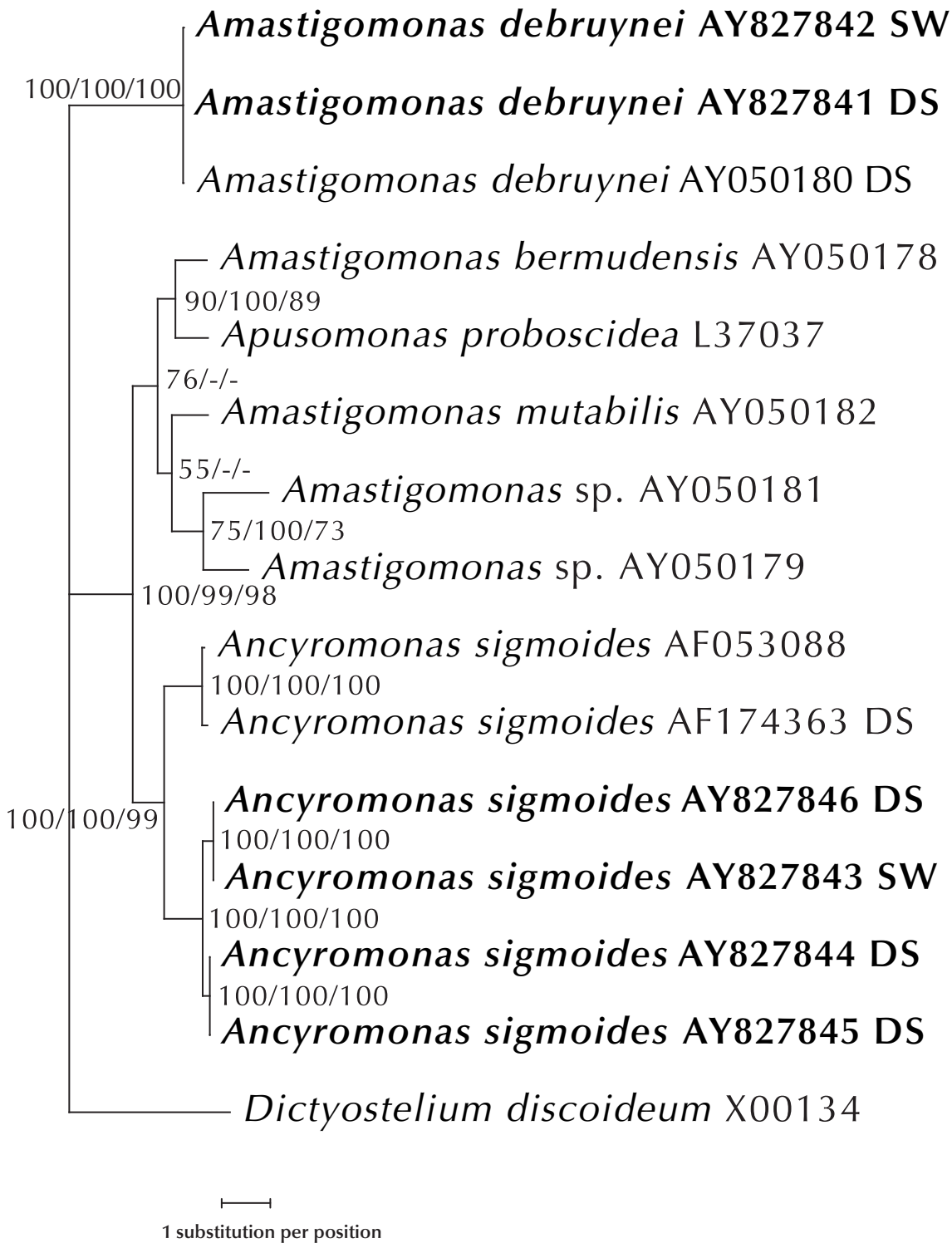

Figure 1.2: Phylogenetic tree of Apusozoa using NJ, MP and ML methods (NJ/ML/MP). Number of informative sites for ML and MP analysis was 597 and 513, respectively. Further details as in Fig. 1.1.

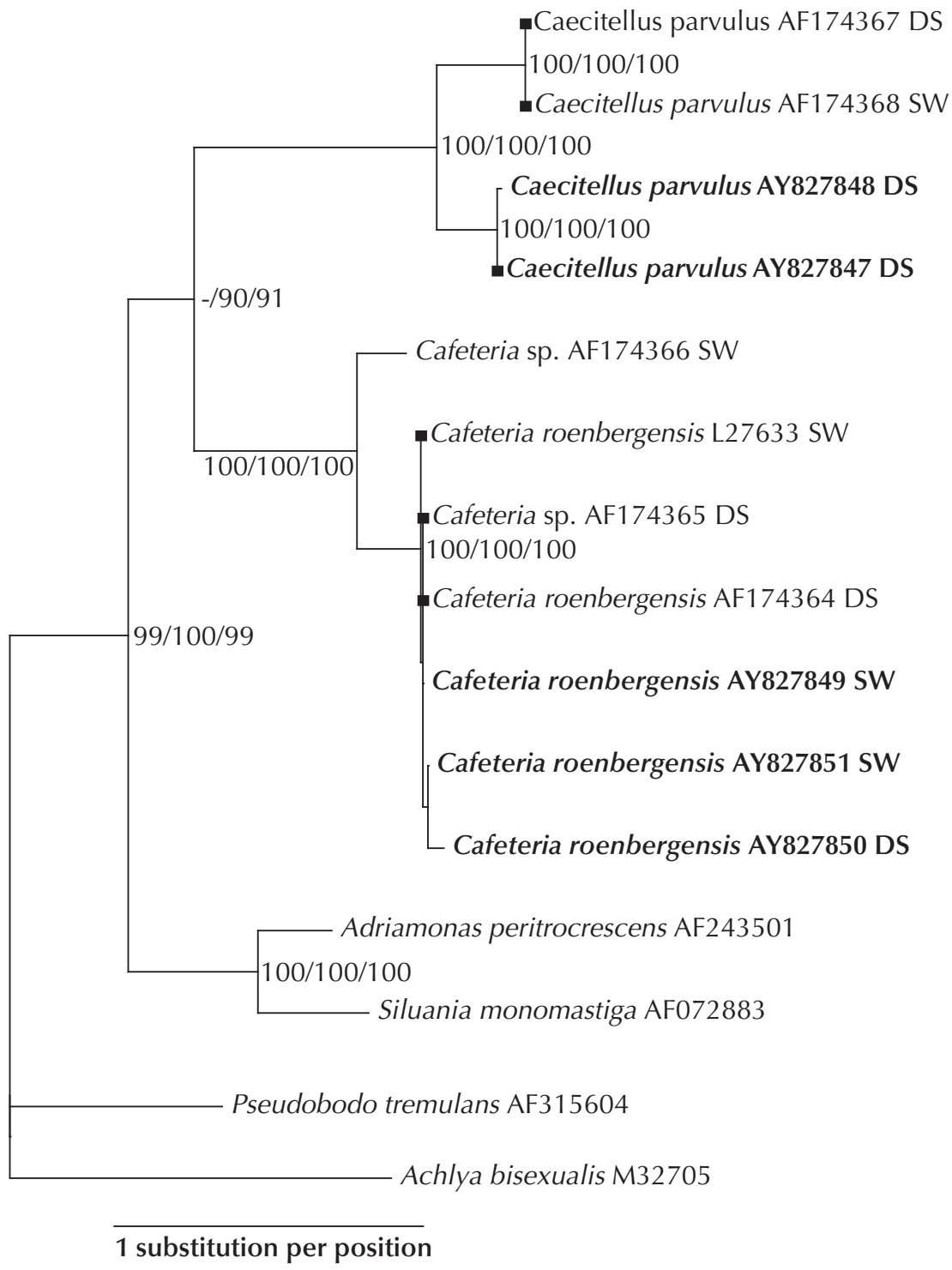

Figure 1.3: Phylogenetic tree of Bicosoecida using NJ, MP and ML methods (NJ/ML/MP). Number of informative sites for ML and MP analysis were 496 and 350, respectively. Further details as in Fig. 1.1.

chomonas 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* AY827855) 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 pyriformis* (Nanney et al. 1998), coccoid green algae (Saéz et al. 2003) or heterotrophic flagellates (Von der Heyden et al. 2004a), 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 1.7) and up to 5 (*Rhynchomonas nasuta*; Table 1.3) similar strains. Genetic variation is comparatively low within these clades, with a maximum sequence divergence of 2.0 % (*Ancyromonas sigmoides* AY827843 and AY827846). 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 of its 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 determine genotype variability of morphospecies, and that our estimates of diversity might 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. 1.1). These distances are also similar for different genera of the Apusozoa (e. g. sequence dissimilarity between *Ancyromonas sigmoides* AF053088 and *Apusomonas proboscidea* L37037 is 12.3 %, Fig. 1.2). Bearing in mind that a divergence of 6.1 % between different strains of *Bodo saltans* has been 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). Where 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 and Nerad (1998).

All 5 species studied are described as bacteriovorous benthic species and are reported to belong to the 20 most abundant species of heterotrophic flagellates with worldwide distribution (Patterson & Lee 2000). Being poor swimmers, their common occurrence in marine pelagic environments points to the existence of specialised microenvironments 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 the a wide range of environmental conditions (Finlay 2002, 2004). In contrast, some protist morphospecies show a clear pattern of geographic distribution (Medlin et al. 2000, Coleman et al. 2001) that cannot be resolved by SSU rDNA sequences but only by more variable regions like the internal transcribed spacers (ITS).

Only a few molecular studies have investigated the 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. López-García et al. 2001, 2003, Díez 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 investigated 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 studies underlines the need for the application of molecular techniques based on rDNA sequencing, which could lead to significant changes in flagellate taxonomy (Caron et al. 2004).

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Part II.

Molecular comparisons of freshwater and marine isolates of the same morphospecies of heterotrophic flagellates

Abstract

Heterotrophic flagellates are key components of all ecosystems. Understanding their patterns of biodiversity is thus of particular importance. However, recent molecular studies have shown, that ecologically relevant patterns can be found within morphologically defined species, suggesting that the morphospecies-concept might be insufficient in assessing the actual biodiversity of heterotrophic flagellates. We have thus sequenced the small subunit ribosomal DNA (SSU rDNA) of several strains isolated from marine- and freshwater-environments, as well as from soil and groundwater, of the morphospecies *Ancyromonas sigmoides, Apusomonas proboscidea, Bodo saltans, Dimastigella mimosa, "Bodo" curvifilus, Neobodo designis, Neobodo saliens, Parabodo caudatus, Procryptobia sorokini* and *Rhynchomonas nasuta*, all belonging to the most common flagellates with world-wide distribution. Our results reveal a clear divergence between marine and freshwater lineages of the morphospecies *A. sigmoides*, showing that ecologically important patterns might be commonly found within flagellated morphospecies and that ecological factors can act as major constraints on speciation. The genetically highly diverging marine lineages of *A. sigmoides* have obviously not been able to colonise freshwater environments for a long time, and vice versa. In contrast, most other morphospecies do not show any environment-specific clustering at present. Mixing of lineages isolated from diverse habitats, indicate that some lineages of these morphospecies have been able to colonise different habitats even several times, showing impressive ecological tolerances. Furthermore, our results reveal remarkable genetic divergence within most morphospecies studied, underlining the difficulties in correctly determine species using morphology alone. There are apparently far more cryptic or pseudo-cryptic species than established morphospecies within the heterotrophic flagellates studied.

Key Words Molecular ecology · Protist · Protozoa · Eukaryotic microbiology · Morphological adaptive peaks · Parallel evolution · Ecotype

Introduction

The application of a morphological species concept in microbial taxonomy has promoted the view of a cosmopolitan distribution of a reduced number of species (Beijerinck 1913, Baas-Becking 1934, Finlay 2002). The introduction of molecular tools into microbial research has started to change this point of view. Molecular studies are not only showing the vast amount of microbial diversity (Pace 1997, Rappé & Giovannoni 2003), but also that there might be micro-organisms with restricted distribution (Papke et al. 2003). Whitaker et al. (2003) showed, that populations of the hyperthermophilic Archaea *Sulfolobus* are geographically isolated from one another. The higher resolution of molecular tools has furthermore revealed an astonishing molecular microdiversity (Giovannoni et al. 1990, Ward et al. 1990, Fuhrman et al. 1993, Fuhrman & Campbell 1998), leading to the suggestion, that microbial species might be considered as microdiverse ribotype clusters (Acinas et al. 2004), possibly representing ecological cohesive populations, or ecotypes (Cohan 2002). It could have furthermore been shown, that genetic clusters within named species of bacteria are ecologically distinct (Field et al. 1997, Maiden et al. 1998, Moore et al. 1998, Zhu et al. 2001). The fine structure of phylogenetic trees can thus hold information on the evolution and ecology of microbial populations (Cohan 2002, Rappé & Giovannoni 2003, Giovannoni & Stingl 2005).

Finding similar patterns of eukaryotic microbial biodiversity is of particular importance, because eukaryotic microbial species (protists) are primary consumers of bacteria and play a major role in the microbial food web (Pomeroy 1974, Azam et al. 1983, Arndt et al. 2000). The criteria for defining most species of protists is morphology. However, an increasing number of studies show that, though protists sometimes have an amazing amount of phenotypical traits compared to prokaryotes, morphology alone often proves to be of limited use in assessing species boundaries (Schlegel & Meisterfeld 2003). This is partly due to the lacking of morphological traits in small protists and to the fact that many protists are polymorphic in the way that they have a variable or amorphous cell shape, or different morphologies according to different life stages. Some traditional protists are actually composed of several genotypic or ecophysiological lineages, just as prokaryotes, which might be referred to as distinct species, and which are masked under the same or very similar morphologies (de Vargas et al. 1999, Darling et al. 2000, Norris & de Vargas 2000, Sáez et al. 2003, Darling et al. 2004). Rodríguez et al. (2005) have found distinct lineages of the protist *Ostreococcus* adapted to the environmental conditions of the different depths of the euphotic zone from where they were isolated, as has been described for the prokaryote *Prochlorococcus* (Moore et al. 1998).

For bacteria, tolerances to high salinity have been recognised as important physiological property that can be used to define a phylogenetic lineage (Hiraishi & Ueda 1994, Nübel et al. 2000). Many species of freshwater bacteria are in general not salinity tolerant (Painchaud et al. 1995) and microbial freshwater communities contain a high proportion of such salinity intolerant bacteria. Moreover, evidence is raising that microbial communities differ significantly between marine and freshwater environments

(Glöckner et al. 2000, Rappé & Giovannoni 2003). Molecular analysis have revealed clades only composed of phylotypes retrieved from one habitat, such such as the marine parts of the SAR clusters (Giovannoni et al. 1990), and freshwater-specific clades have been reported by other authors (Zwart et al. 1998, Warnecke et al. 2004). Environmental factors are thus influencing microbial community composition and species distribution and can be major forces of diversification (Horner-Devine et al. 2003, Kassen & Rainey 2004).

Although many protists show apparently high physiological tolerances, as indicated by the cosmopolitan distribution of many morphological defined named species in different environments (Patterson & Simpson 1996, Tong 1997a, Patterson & Lee 2000), it is expected that evolutionary distinct lineages should also be present in marine and freshwater environments within protists. This is supported by the fact, that physiological differences and specific tolerances of several strains of the cosmopolitan flagellate *Neobodo designis* towards NaCl concentrations might be due to genetic adaptation (Ekelund 2002, Koch & Ekelund 2005). Moreover, Koch and Ekelund (2005) showed that—with 1 exception—at least strains of *N. designis* isolated from freshwater were not able to grow if salinity was above 15 ‰. Strains of the flagellate *Oxyrrhis marina* showed different salinity preferences and salinity tolerance could be associated with habitat type (Lowe et al. 2005). Von der Heyden et al. (2004a) reported a clear divergence between marine and freshwater lineages of the flagellate *Goniomonas*.

Considering the fact that protists can be composed of several genotypes with different ecophysiological preferences, especially towards NaCl concentrations, shouldn't one expect to find a genetic divergence between marine and freshwater lineages in other morphospecies? Cosmopolitan morphospecies, showing a wide ecological plasticity, should be interesting candidates to answer this question. To be able to detect diverging clusters of freshwater and marine lineages, it is necessary that the studied morphospecies contain different genotypes. Thus, we have sequenced the small subunit ribosomal DNA (SSU rDNA) of several heterotrophic flagellates, most of which are known to form large genetic clusters (von der Heyden et al. 2004b, Scheckenbach et al. 2005), with focus on the morphospecies *Ancyromonas sigmoides, Bodo saltans, Neobodo designis* and *Rhynchomonas nasuta*, four species regarded as cosmopolitan and belonging to the 20 most commonly reported species of heterotrophic flagellates (Patterson & Lee 2000), as well as *Apusomonas proboscidea, "Bodo" curvifilus, Parabodo caudatus, Procryptobia sorokini* and *Neobodo saliens* from marine and freshwater sites, as well as from soil and groundwater.

Materials and Methods

Heterotrophic flagellate strain culturing and DNA sequencing

The list of clonal cultures used and their sampling location is given in Table 2.1. Marine strains were isolated in 2000 during the expedition with R/V "Meteor" cruise 48 leg 1 (expedition DIVA 1) in the oligotrophic South Atlantic, Angola Basin, and in 2002

Table 2.1: Sample location of all species sequenced, with accession number for GenBank.

Species name	Accession No.	Sample location
Ancyromonas sigmoides	DQ207563	River Rhine, Cologne, Germany
Ancyromonas sigmoides	DQ207564	River Rhine, Cologne, Germany
Ancyromonas sigmoides	DQ207565	Pond, Zoological Institute, University of Cologne, Germany
Ancyromonas sigmoides	DQ207566	River Rhine, Cologne, Germany
Apusomonas proboscidea	DQ207567	Pond near Borok, Yaroslavl, Russia
Apusomonas proboscidea	DQ207568	Groundwater, -18 m, Bornheim, Germany
"Bodo" curvifilus	DQ207577	Lake Schoehsee, Germany
Bodo saltans	DQ207569	Pond near Borok, Yaroslavl, Russia
Bodo saltans	DQ207570	Sewage plant tank, Stammheim, Germany
Bodo saltans	DQ207571	Black Sea near Gelandchik, Russia
Bodo saltans	DQ207572	Pond, Zoological Institute, University of Cologne, Germany
Bodo saltans	DQ207573	River Rhine, Cologne, Germany
Bodo saltans	DQ207574	Pond, Randwick, Sidney, Australia
Bodo saltans	DQ207575	River Rhine, Cologne, Germany
Dimastigella mimosa	DQ207576	Pond, Borok, Yaroslavl, Russia
Neobodo designis	DQ207578	Brackish water, Baltic Sea near Hiddensee, Germany
Neobodo designis	DQ207579	River Rhine, Cologne, Germany
Neobodo designis	DQ207580	Sheet of ice, 79°00.00 N 2°00.00 W, North Atlantic Ocean
Neobodo designis	DQ207581	Sheet of ice, 79°00.00 N 12°00.00 W, North Atlantic Ocean
Neobodo designis	DQ207582	River Rhine, Cologne, Germany
Neobodo designis	DQ207583	River Rhine, Cologne, Germany
Neobodo designis	DQ207584	Soil, Garden of the Zoological Institute, University of Cologne, Germany
Neobodo designis	DQ207585	Pond, Zoological Institute, University of Cologne, Germany
Neobodo designis	DQ207586	Soil, Bienen, Germany
Neobodo designis	DQ207587	Soil, Grietherbusch, Germany
Neobodo designis	DQ207588	Soil, Millinger Waard, Netherlands
Neobodo saliens	DQ207589	Brackish water, Baltic Sea near Hiddensee, Germany
Parabodo caudatus	DQ207590	River Rhine, Cologne, Germany
Parabodo caudatus	DQ207591	Lake Schoehsee, Germany
Procryptobia sorokini	DQ207592	Pond near Borok, Yaroslavl, Russia
Procryptobia sorokini	DQ207593	Sheet of ice, 79°00.00 N 2°00.00 W, North Atlantic Ocean
Rhynchobodo sp.	DQ207594	Soil, Garden of the Zoological Institute, University of Cologne Germany
Rhynchomonas nasuta	DQ207595	Pond, Borok, Yaroslavl, Russia
Rhynchomonas nasuta	DQ207596	Sediment, 18°25.3' S 4°44.0' E, -5392 m, Angola Basin, South Atlantic
Rhynchomonas nasuta	DQ207597	Sediment, 17°11.6' S 4°45.9' E, -5415 m, Angola Basin, South Atlantic
Rhynchomonas nasuta	DQ207598	River Rhine, Cologne, Germany
Rhynchomonas nasuta	DQ207599	Sediment, 19°06.9' S 3°52.0' E, -5423 m, Angola Basin, South Atlantic

during "Polarstern" cruise 18 leg 1 in the North Atlantic. Clonal strains were established by micromanipulation and kept in culture in 50 ml tissue culture flasks (Sarstedt) with autoclaved artificial sea water (28.15 g NaCl, 0.67 g KCl, 5.51 g $MgCl_2 \cdot 6H_2O$, 1.45 g $CaCl_2 2 \cdot H_2O$, 6.92 g $MgSO_4 \cdot 7H_2O$, 0.1 g KNO_3, 0.01 g $K_2HPO_4 \cdot 3H_2O \cdot l^{-1}$) if marine isolates and otherwise with autoclaved WC media (Guillard & Lorenzen 1972) together with a sterile wheat grain as food for the bacteria. Morphological identification of each strain was based on light microscopy. All strains are referred to by their GenBANK accession number.

The cultured isolates were grown to high densities (10^4 cells $\cdot$ ml^{-1}) and harvested by centrifugation in 50 ml tubes (Sarstedt) for 30 min. at 4000 $\times$ g and 4 °C. The supernatant was discarded and the cells resuspended in 50 µl 1 $\times$ TE buffer and transferred into PCR tubes. Collected cells were lysed and genomic DNA was isolated using a CTAB extraction method with phenol and chloroform as previously described (Clark 1992). Amplification of the SSU rDNA was performed using standard PCR conditions and cycle sequencing was performed following the manufacturer's specifications as described in Scheckenbach et al. (2005) using the same external and internal primers.

Phylogenetic analysis

Determined sequence fragments were checked and assembled manually. The determined sequences were aligned together with other sequences retrieved from GenBANK-/EMBL using the ClustalX multiple alignment program (Version 1.83; Thompson et al. 1994). Uncorrected genetic distances (*p*-distances; Nei & Kumar 2000) were calculated using MEGA Version 3 (Kumar et al. 2004). Phylogenetic analyses were carried out by using a distance method (minimum evolution, ME; Rzhetsky & Nei 1992) and the maximum likelihood method (ML; Felsenstein 1981). The models of nucleotide substitution which best fitted the given data set were estimated with J. A. A. Nylander's MrAIC (Version 1.4; Department of Systematic Zoology, Uppsala University) evaluating 56 different models. Minimum evolution phylogenetic trees were calculated with FastME (Desper & Gascuel 2002) and ML phylogenetic trees with Phyml Version 2.4.4 (Guindon & Gascuel 2003) using all aligned sites. GTR + Γ (e. g. Lanave et al. 1984, Tavare 1986, Rodríguez et al. 1990) distances for ME analysis of Kinetoplastea and TrN + Γ + I (Tamura & Nei 1993) distances for ME analysis of Apusozoa, using substitution rates estimated by Phyml, were calculated with Tree-Puzzle Version 5.2 (Schmidt et al. 2002). Parametric bootstrapping (Felsenstein 1985) with 100 resamplings was performed for ME analysis using Seqboot and Consense from J. Felsenstein's PHYLIP package (Version 3.63; Department of Genetics, University of Washington). Non-parametric bootstrapping with 100 resamplings was performed for ML analysis as implemented in the Phyml program.

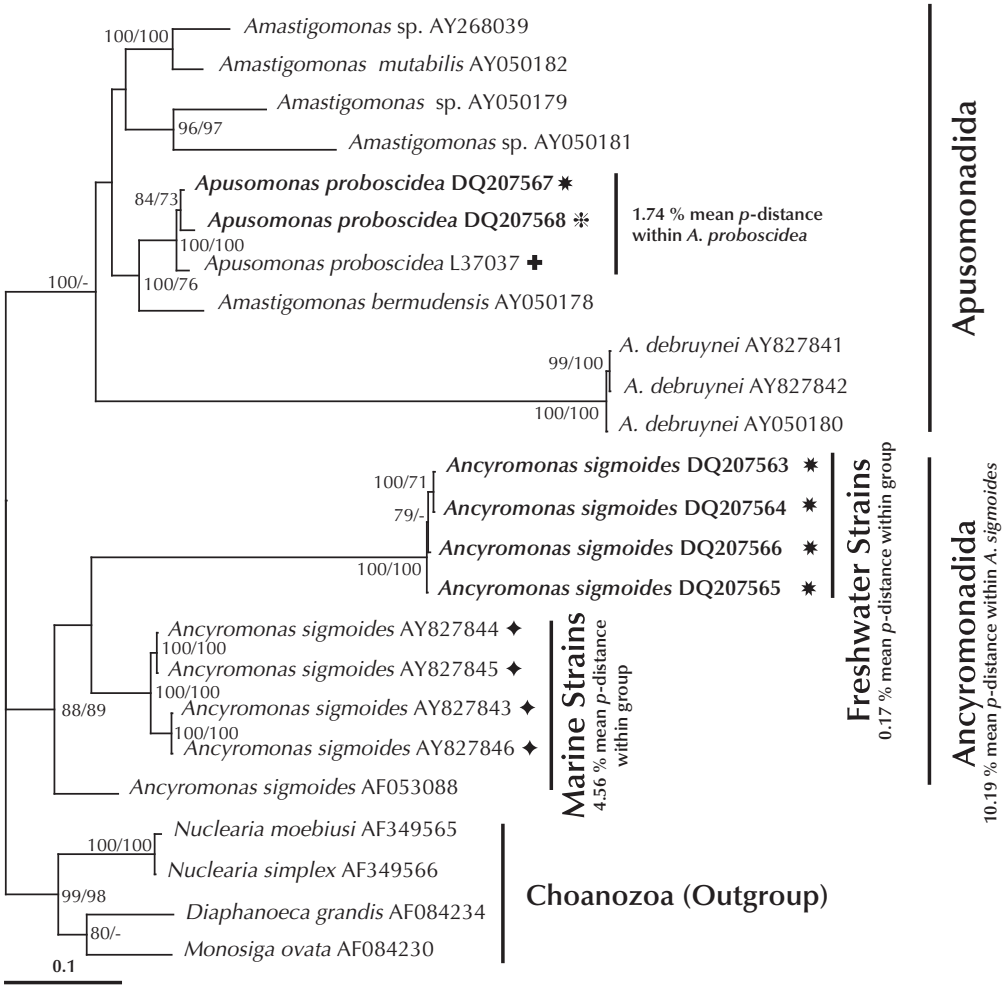

Figure 2.1: Maximum likelihood tree of the phyla Apusozoa using 2037 positions. The tree was rooted using Choanozoa as outgroup. Numbers at the nodes are bootstrap support percentages from 100 replicates for maximum likelihood (left) and minimum evolution (right). Values less than 70 % are omitted or marked by -. Model of nucleotide substitution chosen was $\mathrm{TrN} + \Gamma + I$ with $\alpha = 0.29$. GenBANK accession numbers are given. Strains sequenced in this study are in **bold**. The scale bar represents 0.1 substitutions per site. Freshwater and marine clades of *Ancyromonas sigmoides* are labelled. Mean *p*-distances of the species studies (*Ancyromonas* and *Apusomonas*) and of the major clades within *A. sigmoides* are shown. Sample locations within morphospecies studied are labelled as follows: ✦ marine, ✷ freshwater, ✚ soil, ❊ groundwater.

Results and Discussion

Marine and freshwater lineages of *Ancyromonas sigmoides*

There is a complete phylogenetic separation between the marine and the freshwater lineages of the morphospecies *Ancyromonas sigmoides* (Fig. 2.1). Freshwater-specific lineages are ancestrally related with but phylogenetically distinct from the marine lineages with high genetic divergence between them (16.16 % mean *p*-distance between the five marine strains and the freshwater strains; 0.17 % mean *p*-distance within freshwater cluster). The freshwater cluster is stable with all phylogenetic methods used and clearly separated from the marine lineages by bootstrap support values of 100 % and long evolutionary branches.

Regarding the high divergence between the phylogenetically coherent marine and freshwater lineages of *A. sigmoides*, it is obvious that populations of one habitat have not colonised the other for a long time. The topology of the *Ancyromonas*-cluster suggests an evolutionary divergence into different ecotypes—possibly species. Since dispersal rates for this morphospecies should be as high as for other microbial species, one should expect to find marine lineages in freshwater-environments and vice versa (Finlay 2002, Griffin et al. 2002). The observed pattern must be the result of different ecophysiological traits (Finlay 2004)—or a kind of iceberg bias due to undersampling. The ecological differences between marine and freshwater habitats are therefore sufficient barriers of dispersal for at least some lineages of *A. sigmoides*, as was previously reported for *Goniomonas* (von der Heyden et al. 2004a), restricting the geographical distribution of the respective lineages. The environment obviously selects for very particular phylogenetic clusters and thus ecophysiological differentiation below the morphospecies level, though it is not possible to deduce from the phylogenetic position which abiotic or biotic factors determine the distribution of the corresponding lineages between the respective habitats. Taking salinity tolerance as the only decisive factor for the distribution might be wrong since at least *Goniomonas pacifica*, which is only reported from marine environments, was able to tolerate salt concentrations from 35 ‰ down to 0 ‰ (Arndt et al. 2000). Either *G. pacifica* is outcompeted in freshwater habitats or other factors might play an important role in the distribution of this morphospecies and its selective disadvantage in freshwater habitats as shown by von der Heyden et al. (2004a).

Successive occurrences between environments

The morphospecies *Bodo saltans, Neobodo designis, Parabodo caudatus, Procryptobia sorokini* (*Parabodo sorokini* is actually a synonym for *Procryptobia sorokini*) and *Rhynchomonas nasuta* contain strains clustering amongst other strains isolated from different habitats (Fig. 2.2). The marine strains of *B. saltans* (DQ207571 and AY490233) cluster between freshwater, freshwater-sediment and soil isolates. Freshwater, freshwater-sediment and soil isolates of the same morphospecies cluster together. Soil isolates of *P. caudatus* and *N. designis* cluster together with freshwater isolates of these morphospecies. Marine isolates of *P. sorokini* and *R. nasuta* cluster together

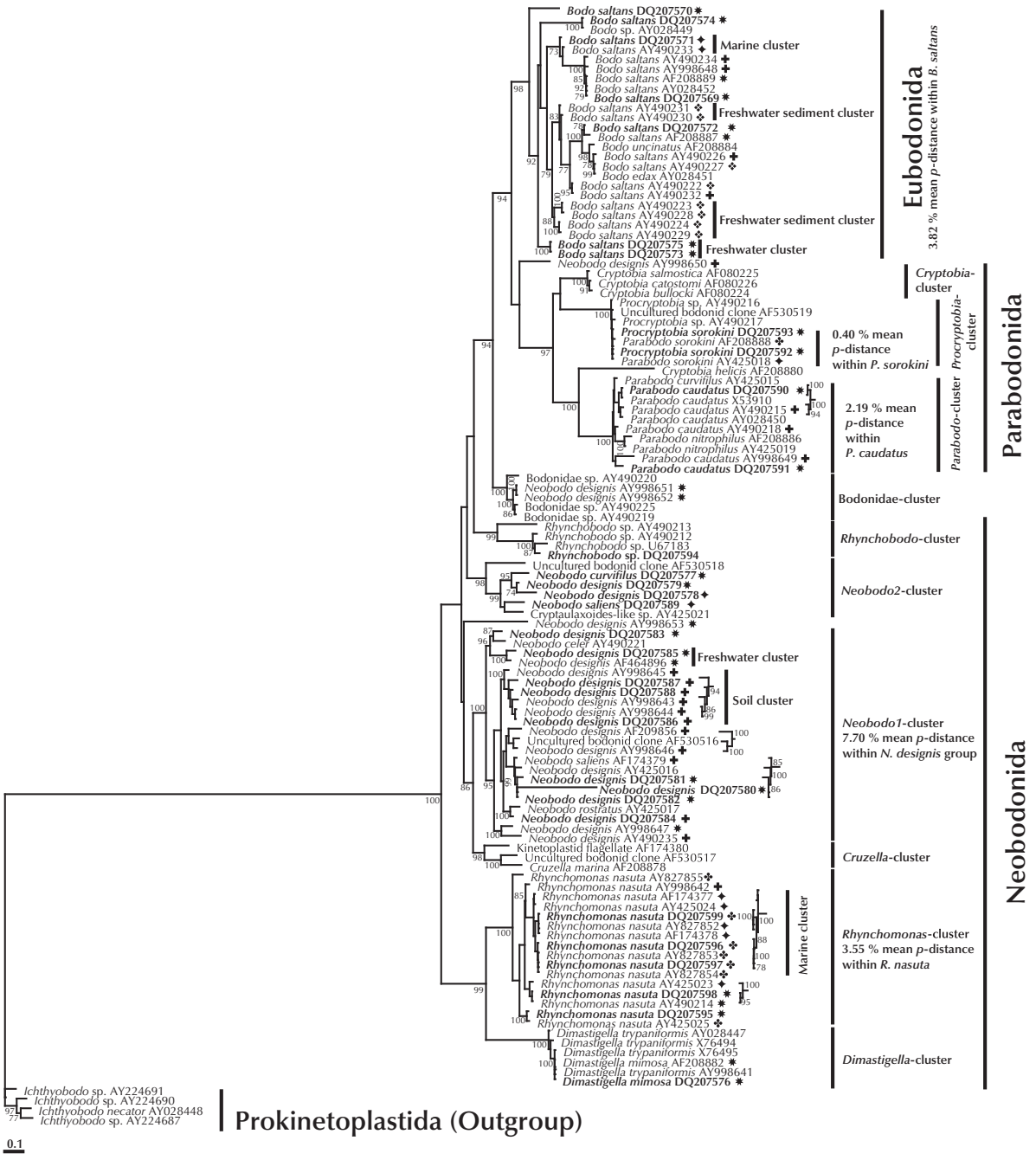

Figure 2.2: Maximum likelihood tree of the class Kinetoplastea using 2383 positions. The tree was rooted using Prokinetoplastida as outgroup. Model of nucleotide substitution chosen was GTR + Γ with $\alpha = 0.41$. The scale bar represents 0.1 substitutions per site. Some clades are labelled according to their sample location. Mean *p*-distances of all mono- and paraphyletic species studied and of the major clade of *N. designis* are shown. Sample locations within morphospecies studied are labelled as follows: ✦ marine, ✷ freshwater, ✚ soil, ✤ marine-sediment, ❖ freshwater-sediment. Further details as in Figure 2.1.

with freshwater isolates, and one soil strain of *R. nasuta* (AY998642) clusters between marine and freshwater strains. Clustering of strains is thus not environment-specific within these morphospecies and the succession of strains according to their sample locations alternates in the phylogenetic trees within one morphospecies. These taxa have obviously been able to move across ecosystem boundaries and even to colonise several habitats multiple times.

Ekelund (2002) supposed, that the ability to tolerate varying salt concentrations, what is supposed to be the most important characteristic for successful immigration from marine to freshwater environments and vice versa, is rather not the property of the morphospecies but of the respective strains. The morphospecies mentioned above, having colonised marine and freshwater habitats multiple times, must thus at least have contained true euryhaline strains, which must have been able to tolerate varying salt concentrations. This has been shown for a marine strain of *N. designis* (AY998646), as well as one soil strain of the same morphospecies (AY998645), which could survive and grow at salinity ranges from 0 ‰ to 45 ‰ (Koch & Ekelund 2005). This is furthermore in accordance with previous findings (Arndt et al. 2000), where marine strains of *B. saltans* and *N. designis* could have been transferred slowly and gradually into salt concentrations of 0 ‰ and vice versa a freshwater strain of *B. saltans* into salt concentrations of 35 ‰.

Identical genotypes of the same morphospecies isolated from different habitats

Rhynchomonas nasuta DQ207599 from sediments of the Angola Basin and sequence AF174378 from a biovent serpulid zone of the east Pacific Rise are identical (0.00 % *p*-distance). A previous study has already reported 1 identical pair of strains from different sample locations of *Ancyromonas sigmoides* (AY827843 from brackish water of the Baltic and AY827846 from deep sea of the North Atlantic Ocean; Scheckenbach et al. 2005). Some taxa might thus be able to move across ecosystem boundaries on a global scale and show a high degree of ecotypic flexibility. But this might quite well just be the result of our inability to recognise the actual nature of their microhabitats, respectively their ecological niches, and due to the insufficient resolution of the SSU rDNA at or below the species level (Avise 2004).

For bacteria it is known that even phylogenetically identical strains can represent different ecotypes (Lebuhn et al. 2000, Jaspers & Overmann 2004). Protists with identical SSU rDNA show high genetic divergence if more variable markers such as the ITS are used (Tsuchiya et al. 2003) and can have different ecophysiological characteristics as has been shown for the heterotrophic flagellate *Oxyrrhis marina* (Lowe et al 2005) and the prasinophycean *Ostreococcus* (Rodríguez et al. 2005). Phylogenetic markers and especially slowly evolving markers such as the SSU rDNA might thus not be sufficient to detect ecophysiological differences between populations of the same named species. Regarding the different ecological environments from where identical strains of *A. sigmoides* and *R. nasuta* have been isolated, one should expect that there might be functional differences that have ecological significance.

Strains of Kinetoplastea isolated from the same habitat clustering together

Within some morphospecies, clusters of similar or identical strains from the same habitat-type are present (Fig. 2.2). With the exception of the *Bodo saltans* freshwater clade, which is composed of both strains isolated from the river Rhine at Cologne (DQ207573 and DQ207575), all strains of these clusters have been isolated from geographically different locations and all are phylogenetically distinct from other strains of the corresponding morphospecies. There are several such environment-specific clades of freshwater-sediment, freshwater and marine isolates within *Bodo saltans*. Analogue, a freshwater and soil cluster exists in *Neobodo designis* and a large cluster of exclusively marine isolates is present within *Rhynchomonas nasuta*.

Whether these clusters are just the result of undersampling or might turn out in the future to form distinct lineages with different ecological characteristics, can not be said up to now. Microdiversity might be of ecological relevance since phylogenetically coherent lineages are believed to be ecophysiologically distinct from other lineages (Cohan 2002, Finlay 2004), and cosmopolitan species present in a multitude of different habitats should contain ecologically distinct lineages (T. Weisse, personal communication).

High genetic divergence

The branching order between Neobodonida and both Eubodonida and Parabodonida, as well as between the 6 major neobodonid clusters (*Cruzella*-cluster, *Neobodo1*-cluster, *Neobodo2*-cluster, *Rhynchobodo*-cluster, *Rhynchomonas-Dimastigella*-cluster) differs between ML and ME analysis (Figs. 2.2 & 2.3), but all clusters within Parabodonida (*Cryptobia*-cluster, *Parabodo*-cluster and *Procryptobia*-cluster) and Neobodonida, as well as the eubodonid cluster, are stable with both phylogenetic methods used and with high bootstrap support values ($\geqslant$ 96 %; Figs. 2.2 & 2.3). The apusozoan *Ancyromonas sigmoides* forms a coherent phylogenetic cluster (bootstrap value of 88 % in ML and 89 % in ME analysis; Fig. 2.1), as does *Apusomonas proboscidea* (bootstrap value of 100 %; Fig. 2.1).

The genetic divergence, given as *p*-distance, is generally very high within most morphospecies studied, as shown for some taxa studied and some of the major intraspecific clusters in Figs. 2.1 & 2.2. In addition, pairwise *p*-distance of *"Bodo" curvifilus* (DQ207577 and AY425015, which might rather be *Parabodo caudatus*-like; von der Heyden et al. 2004b) is 14.30 % and of *Neobodo saliens* 11.81 %. Mean *p*-distance of *Neobodo designis* is 9.64 %. These are really high values considering that *p*-distances are not corrected for multiple base changes at individual nucleotide positions.

With the exception of *A. proboscidea, A. sigmoides, P. sorokini* and *R. nasuta*, all of the morphospecies sequenced in this study are para- or even polyphyletic. High levels of para- and polyphyletic taxa within the Kinetoplastea, such as the genus *Cryptobia* (Dolezel et al. 2000) or the species *Neobodo designis* (Koch & Ekelund 2005), were well known and have already led to a replacement of the former genus *Bodo* by 3 genera: *Bodo sensu stricto*, *Neobodo* and *Parabodo* (Moreira et al. 2004). Despite, taking species of the genus *Cryptobia* apart, at present 7 (*B. saltans, D. mimosa, Dimastigella trypaniformis, P. caudatus, "Bodo" curvifilus, N. designis* and *N. saliens*) out of 15 morphospecies within the Kinetoplastea are still para- or polyphyletic (Figs.

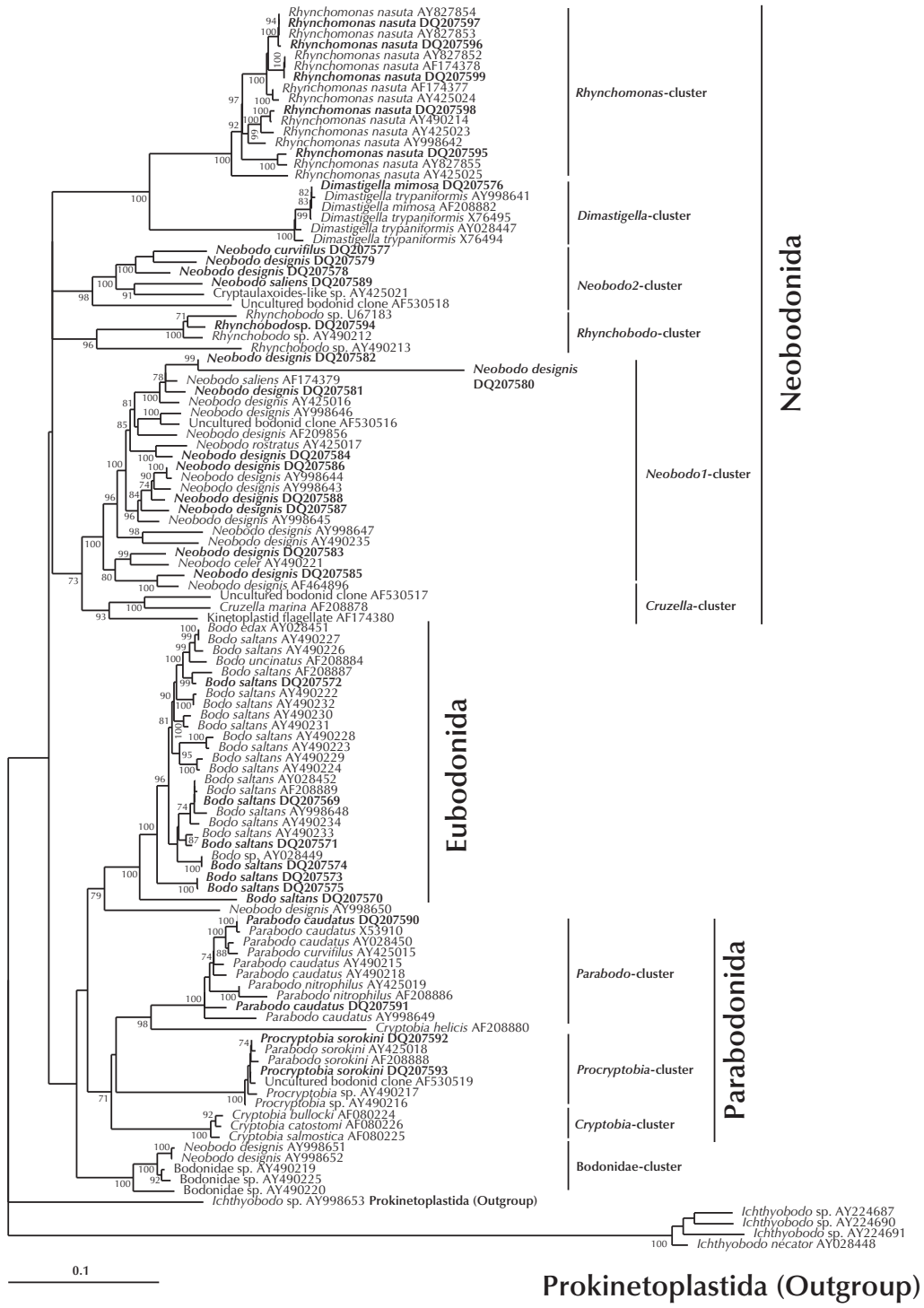

Figure 2.3: Minimum evolution tree of the class Kinetoplastea using 2383 positions. The tree was rooted using Prokinetoplastida as outgroup. Model of nucleotide substitution chosen was GTR + Γ with $\alpha = 0.41$. The scale bar represents 0.1 substitutions per site. Further details as in Figure 2.1.

2.2 & 2.3). This is amazing, if one keeps in mind that, irrespective of the species concept applied, every species must at least fulfil the criteria of monophyly. High degrees of species-level para- and polyphyly have also been observed in animal mitochondrial DNA and might be a much more important phenomenon than generally recognised (Funk & Omland 2003). The difficulties in identifying species within the genera *Bodo* and *Neobodo*, especially using light microscopy, are well known and might have led to a misidentification of some strains such as *Bodo edax* (AY028451), *Bodo uncinatus* (AF208884) or *Neobodo saliens* (DQ207589 and AF174379) as pointed out by von der Heyden et al. (2004b), and many taxa within the Kinetoplastea might therefore be overlumped.

There is no general rule on the level of genetic divergence needed to define species boundaries in protists and the level might change from species to species. Taking 0.50 % divergence as rough guideline (T. Weisse, personal communication), all of the morphospecies studied must contain cryptic species—with the exception of *D. mimosa* and *P. sorokini*. And since *D. mimosa* is paraphyletic, only one of the morphospecies sequenced in this study remains which might be at present valid: *P. sorokini*.

Cryptic species might be the result of parallel evolution (Simpson 1961, Zhang & Kumar 1997, Futuyma 1998, Wood et al. 2005) towards adaptive peaks in morphotypes (Nanney 1982). This seems to be rather common within the Kinetoplastea and possibly within the Apusozoa, as was shown within the foraminiferan family Nummulitidae (Holzmann et al. 2003). Alternatively, the phenotypes of cryptic morphospecies, such as the polyphyletic *N. designis*, might be old forms representing early adaptive peaks (Nanney 1982, Koch & Ekelund 2005) and all descendent lineages might still share these morphological traits, as has been suggested for the species complex of the ciliate *Tetrahymena pyriformis* (Nanney 1982).

Conclusion

It appears that the habitat selects very particular phylogenetic lineages of some protists, such as *Ancyromonas sigmoides*. The genetic structure of *A. sigmoides* does not seem to be random at all and strongly suggest that selection acts. According to the high dispersal rates of microbes (Griffin et al. 2002), all lineages should be present in both marine and freshwater environments (Finlay 2002). Since the expected occurrence of marine and freshwater lineages of *A. sigmoides* in sympatry could not have been observed, marine and freshwater habitats obviously form different ecological niches and should therefore harbour different species of *Ancyromonas*, moreover since speciation is most likely to occur along steep ecological gradients (Dieckmann & Doebeli 1999). The distinct lineages of the morphospecies *A. sigmoides* must thus be ecophysiologically different and representing different ecotypes (Cohan 2002, Finlay 2004) or species. What makes the difference between the marine and freshwater lineages of *Ancyromonas* and whether the freshwater lineages should be renamed (e. g. *Ancyromonas limnsigmoides*) is currently under examination. Pseudo-cryptic speciation (Sáez et al. 2003) in heterotrophic nanoflagellates has also been observed within *Caecitellus* (Selchow et al., submitted).

Whether the environment-specific clusters present in the other morphospecies studied will persist further sampling is unclear. According to the niche exclusion principle, dif-

ferent ecological niches are supposed to select for specific ecologically and genetically distinct lineages or sequence-similarity clusters (Palys et al. 1997, Cohan 2002, Finlay 2004). Distinct lineages of the same species, isolated from the same habitat, might therefore represent different species. Most taxa of the Kinetoplastea studied contain SSU rDNA sequences isolated from different habitats which fall into numerous and to some extent deeply branching clades. This mixing of strains from different environments shows that these taxa have been able to colonise various habitats several times. The high genetic divergence within these taxa might be the result of their evolutionary radiation across several habitat types.

If this microdiversity will gain further ecological relevance, as is likely and generally assumed, then the species problem of eukaryotic microbial species (Schlegel & Meisterfeld 2003) must receive new attention, especially with view on ecology (Finlay 2004). In particular autecological studies of model organisms must therefore be accompanied by molecular data and assessment of the intraspecific diversity of micro-organisms and—beyond—integrating molecular techniques into taxonomy (Tautz et al. 2003, Blaxter 2004, Dayrat 2005) becomes crucial for ecological studies (Caron et al. 2004, Caron 2005).

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Part III.

Morphospecies of heterotrophic flagellates: A case study for *Caecitellus* spp.

Abstract

Recent molecular studies revealed quite different genotypes within morphospecies of heterotrophic nanoflagellates (HNF) identified by light microscopy, e. g. for *Caecitellus parvulus*, known as one of the 20 most common heterotrophic flagellates world-wide. We combined molecular and morphological analyses to clarify if the morphospecies *Caecitellus parvulus*—as a case study of HNF—includes genetically as well as ultrastructurally distinguishable species with or without a different geographical distribution. Therefore we compared the ultrastructure and the small subunit of the ribosomal DNA (SSU rDNA) of 2 strains of *C.* cf. *parvulus* isolated from deep-sea sediments and from the surface water of the oligotrophic Angola Basin, South Atlantic. The reconstruction of the kinetid architecture of 2 strains of *C.* cf. *parvulus* revealed differences in the number of microtubules in flagellar root 3, which surrounds the oral region and forms the cytoskeleton of the feeding basket. The number of microtubules in this region is also different to the description given earlier in the literature for *Caecitellus parvulus*. The results of the present molecular comparison of the SSU rDNA of 11 different strains of *Caecitellus* propose at least 3 distinguishable species. Our results indicate pseudo-cryptic speciation within the morphospecies *Caecitellus parvulus*. We describe 2 new *Caecitellus* species, i. e., *Caecitellus paraparvulus* and *Caecitellus pseudoparvulus*, newly established within a *Caecitellus* complex.

Keywords Heterotrophic flagellates · *Caecitellus* · species complex · cryptic species

Introduction

Heterotrophic flagellates are major consumers of bacteria, cyanobacteria and microalgae in a large variety of aquatic ecosystems. Consequently, they play an important role as nutrient remineralizers and are mainly responsible for the carbon transfer to higher trophic levels in both pelagic and benthic environments of the oceans (e. g. Fenchel 1982, Azam et al. 1983, Gasol & Vaqué 1993). In natural planktonic assemblages the abundances of heterotrophic flagellates range from 10^2–10^5 cells · ml^{-1} (Berninger et al. 1991). Despite the high abundance and their importance in aquatic ecosystems, the knowledge about the biogeography and species-level diversity of many heterotrophic nanoflagellates is poorly known (Preisig et al. 1991, Lee & Patterson 1998, Arndt et al. 2000). The question of the biodiversity and distribution of global free-living protists is in general still intensively discussed (e. g. Foissner 1999, Finlay 2002). Some studies were carried out to clarify the question of ubiquitous dispersal or endemism of protozoa species by investigations of extreme habitats like the deep sea or even hydrothermal vents (Atkins et al. 2000, Hausmann et al. 2002, Arndt et al. 2003, Scheckenbach et al. 2005). Whereas the deep sea as an extreme habitat (high pressure, absence of light, poor nutrients concentration, low temperature) covers more than 60 % of the earths surface, the knowledge on deep-sea organisms, especially protists, is still very limited (Finlay 2002, Turley 2002). Numerous protists found in deep-sea sediments are known from surface waters, but there are also some protozoa, which were found in the deep sea and which have not been reported from shallow waters (Hausmann et al. 2002, Arndt et al. 2003). There are several mechanisms known, which could be the reason for genetic exchange between protist populations from different habitats. For example the high probability of dispersal of small organisms by e. g. global oceanic circulation, the possibility to form resting stages, or the formation and sinking of marine snow (Finlay 2001, 2002, Turley 2002). Small sinking aggregates constitute micro-environments for many heterotrophic flagellates within the water column (Caron 1991, Turley 2002, Kiørboe et al. 2004), and also for *Caecitellus parvulus*.

The different views of the biodiversity of protists are tightly connected to the different ways of understanding what a species is (Schlegel & Meisterfeld 2003). According to Mayden (1997), there are over twenty different species concepts. The α-taxonomy of heterotrophic flagellates is mostly based on the morphospecies concept (Patterson & Lee 2000). As a result of the small size of flagellates, electron microscopy has to be used for morphological taxonomic characterisation, and beyond, to detect restricted geographical distribution (Foissner 1999), but it is still not used for most field studies and species descriptions. The application of molecular criteria suggests that behind traditional morphospecies a much greater number of physiological or molecular species is hidden (Patterson & Lee 2000).

The heterotrophic nanoflagellate *Caecitellus parvulus* (Griessmann 1913) Patterson et al. (1993) is one of the 20 most common heterotrophic flagellates world-wide (Patterson & Lee 2000) and has always been regarded as a single species (Patterson & Simpson 1996, Larsen & Patterson 1990, Ekebom et al. 1995/1996, Atkins et al. 2000, Al-Qassab et al. 2002, Lee et al. 2003). However, Scheckenbach et al. (2005)

detected different genotypes within the morphospecies. The small biflagellate gliding cells inhabit sediments and particle surfaces. Their anterior flagellum beats stiffly from side to side as cells glide with the posterior flagellum trailing behind. The species was first assigned to the genus *Bodo* as *Bodo parvulus* (Griessmann 1913), but Patterson et al. (1993) revealed ultrastructural features which are not compatible with a bodonid flagellate and placed it in the new genus *Caecitellus*, which they regarded as a genus of uncertain affinities. O'Kelly and Nerad (1998) reconstructed the kinetid architecture of this species and found a high similarity to the Bicosoecida.

Molecular studies from Scheckenbach et al. (2005) revealed quite different genotypes within morphospecies of heterotrophic nanoflagellates, identified by light microscopy. Therefore the studied strains of *Caecitellus parvulus* which were collected during an expedition with RV Meteor (cruise 48/1, DIVA I, year 2000) from deep-sea sediments and surface waters of the oligotrophic South Atlantic, Angola Basin, are designated as *C.* cf. *parvulus*.

The goal of the present study was to clarify if the morphospecies *Caecitellus parvulus* includes genetically, as well as morphologically, and from their behaviour distinguishable species, and whether these species have different geographical distributions. Therefore, we compared the morphology and ultrastructural architecture, their behaviour, as well as the genotype, of 2 strains of *C.* cf. *parvulus*, i. e. an isolate from the deep sea with a strain from the surface water. Furthermore we reconstructed the kinetid architecture of both *C.* cf. *parvulus* strains and compared it with the kinetid of the organism described as *C. parvulus* (Griessmann 1913) Patterson et al. (1993) which is reconstructed by O'Kelly and Nerad (1998). For more detailed answers on the question of biogeographical distribution and biodiversity, we sequenced some more strains of *C.* cf. *parvulus* of the South Atlantic deep sea and one strain, which was isolated from the North Atlantic, and light microscopically described by O'Kelly and Nerad (1998).

Material and Methods

Organism samples and cultivation

All species of *Caecitellus* were collected in July 2000 during the expedition with Meteor cruise 48/1 (DIVA I) in the oligotrophic South Atlantic, the Angola Abyssal Plain (a detailed overview is given in Table 3.1). Clonal cultures were established and kept in culture as described in detail in Scheckenbach et al. (2005). The strain with the GenBANK accession number DQ230538 has been retrieved from the "American Type Culture Collection" (ATCC50091) and was subject to previous analysis by O'Kelly and Nerad (1998). For the ultrastructural studies the strains *Caecitellus* cf. *parvulus* (DQ220712; deep sea) and (DQ220713; surface water) were cultured at 19 °C in artificial seawater (23 ‰). Sterilised wheat grains were added as polysaccharide supply. *Pseudobodo tremulans* (DQ220718) was isolated by A. P. Mylnikov from brackish water of the Baltic Sea. All strains sequenced in this study and all sequences retrieved from GenBANK are referred to by their GenBANK accession numbers.

Table 3.1: GenBANK accession number, source of isolation and length of 18S rDNA of *Caecitellus* strains sequenced in this study.

Accession No.	Source	Sequence length
DQ220712	19°17.4'S 3°52.2'E, -5424 m, Angola Abyssal Plain, South Atlantic Ocean	1631
DQ220713	17°04.9'S 4°40.8'E, -1 m, South Atlantic Ocean	1646
DQ220714	19°17.4'S 3°52.2'E, -5424 m, Angola Abyssal Plain, South Atlantic Ocean	1669
DQ220715	19°19.8'S 3°55.6'E, -5425 m, Angola Abyssal Plain, South Atlantic Ocean	1681
DQ220716	16°23.1'S 5°27.0'E -5388 m, Angola Abyssal Plain, South Atlantic Ocean	1684
DQ220717	18°25.3'S 4°44.0'E, -5392 m, Angola Abyssal Plain, South Atlantic Ocean	1676
DQ230538	Sargasso Sea, -100 m, North Atlantic Ocean	1696

Light Microscopy

Observations were made using an inverted microscope (Zeiss Axiovert 200, equipped with differential interference contrast optics). Micrographs were made on 64 and 200 ASA colour slide films (Kodak elite chrome) using an Olympus OM-2N camera.

Scanning electron microscopy

Cells were fixed for 15 minutes at room temperature on 0.1 % Poly-L-Lysine coated cover slips applying the Parducz fixative (Parducz 1967). Fixed cells were washed 5×5 minutes in artificial sea water (23 ‰), were dehydrated in a graded series of ethanol. Finally, cells were dried in a BAL-TEC CPD 030. After coating with gold in a Balzers Union SCD 040, cells were examined with a FEI Quanta 200 SEM.

Transmission electron microscopy

Cells were concentrated by centrifugation (200 rpm) and fixed for 30 minutes at room temperature in a fixative basically described by O'Kelly and Nerad (1998). After fixation the cells were transferred into agar for better handling, dehydrated in a graded ethanol series, and embedded in araldit epoxy resin. Sections were made with a diamond knife, mounted on formvar-coated grids or slots and stained with uranyl acetate and lead citrate. They were examined with a Philips EM 208 or a 120 Bio Twin TEM.

DNA extraction, SSU rDNA amplification and sequencing

DNA was extracted using a modified CTAB procedure (Clark 1992). The small subunit rDNA was amplified and sequenced as described in detail in Scheckenbach et al. (2005).

DNA sequence analysis

Determined sequence fragments were assembled manually and unambiguously aligned together with other sequences retrieved from GenBANK using ClustalX Version 1.83 (Thompson et al. 1994). Uncorrected genetic distances (*p*-distances) were calculated using MEGA Version 2.1 (Kumar et al. 2001) with pairwise-deletion option set. Phylogenetic analysis were performed by using the maximum likelihood (ML; Felsenstein 1981), maximum parsimony (MP; Swofford & Olsen 1990) and minimum evolution (ME; Rzhetsky & Nei 1992) methods. The reliability of internal branches was assessed by bootstrapping (Felsenstein 1985). For ML analysis the transition/transversion ratio was set to 1.24. The model of nucleotide substitution used for ME analysis was

LogDet (Steel 1994, Lockhart et al. 1994). For ML and MP analysis PHYLIP Version 3.63 (Felsenstein 2004), for ME analysis MEGA Version 3.0 (Kumar et al. 2004) were used.

Results

Overall structure

Caecitellus cf. *parvulus* from the deep sea (DQ220712) and from the surface water (DQ220713) are biflagellated cells and have a slightly rounded triangular profile (Figs. 3.1–3.3). The body shape appears angular, because of a large feeding-basket protruding on the ventral-apical side (Figs. 3.1–3.3). The basket is internally supported by a horseshoe-shaped cytoskeletal structure, made of microtubules, being mainly responsible for the characteristic form of the mouth-region. The large ingestion apparatus is easy to observe, even under light microscopical conditions (Figs. 3.2 & 3.3). SEM micrographs show clearly that a lip surrounds the rim of the oral apparatus (Figs. 3.4–3.6). The cell length varies from 2–4.5 μm. 2 flagella of unequal length originate from the apical-ventral side of the cell (Figs. 3.1–3.6). The anterior flagellum is projecting forward and beats in a stiffly manner laterally. This flagellum is, in both strains of *Caecitellus*, about 1–1.5 × the length of the cell body and is acronematic (Fig. 3.6). The acronomatic part usually takes up $\frac{1}{3}$–$\frac{1}{2}$ of the total flagellar length. Most of the time,

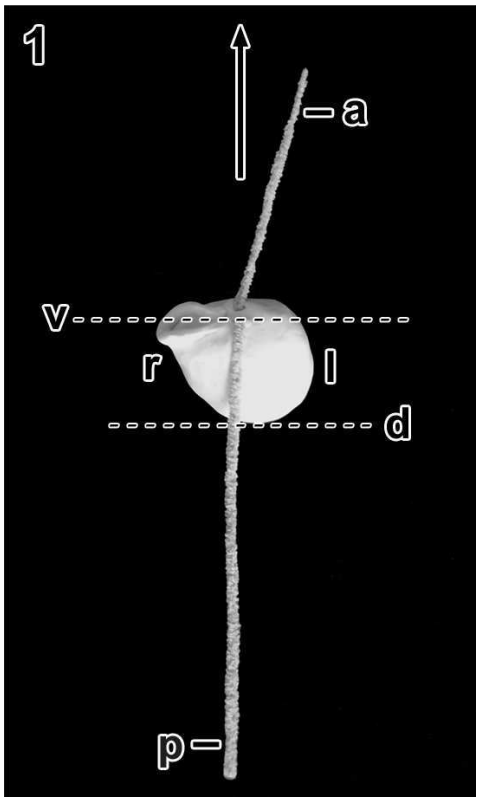
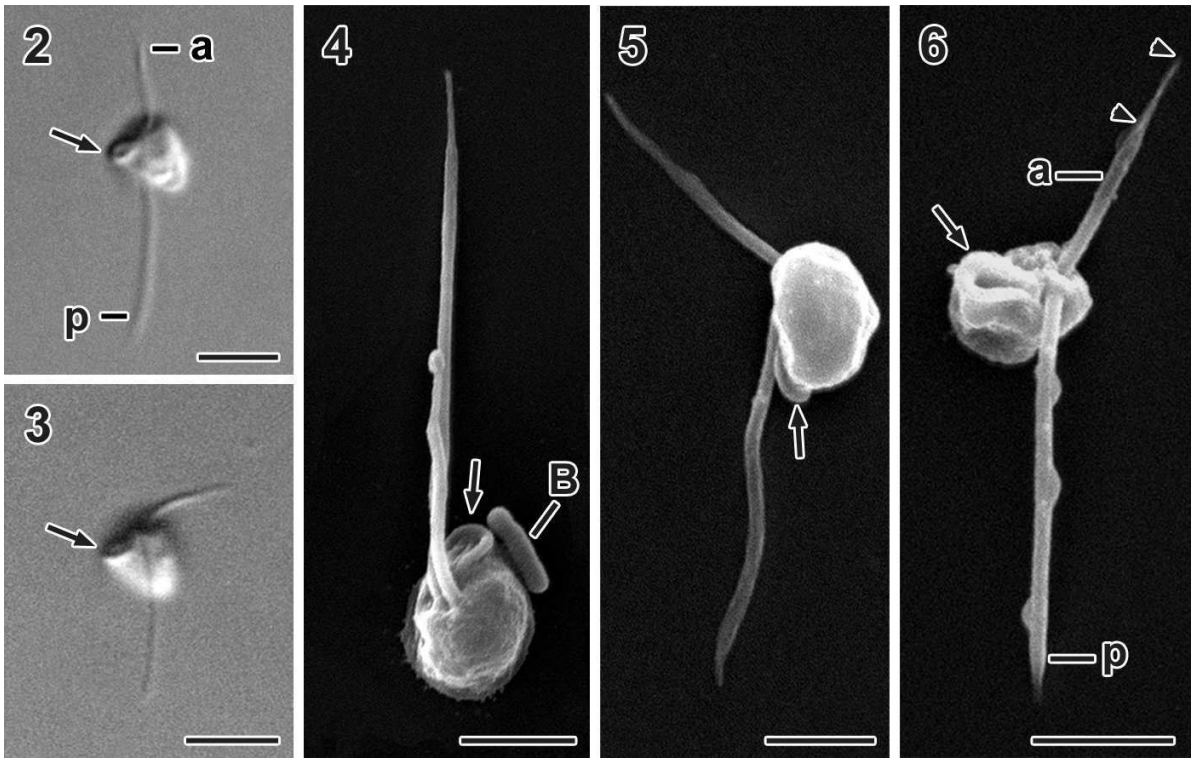


Figure 3.1: Model of *Caecitellus* shows for better orientation the nomenclature of directions. It is marked the anterior flagellum (a), posterior flagellum (p), the ingestion apparatus (arrow) and the directions: R = right, L = left, V = ventral and D = dorsal.



Figures 3.2–3.6: Differential interference contrast light micrographs, ventral view of living cells (**3.2** deep sea strain DQ220712 and **3.3** surface water strain DQ220713), showing the ingestion apparatus at the right side of the cell (arrow). **3.2** Anterior flagellum (a) pointing in direction of locomotion and trailing posterior flagellum (p). Figs. **3.4–3.6**. Scanning electron micrographs showing a lip surrounding the rim of the oral apparatus (arrow). **3.4** Cell of strain DQ220712 with an adjacent bacterium (B). **3.5** Lateral view of a strain DQ220713 cell showing the lip. **3.6** Cell of strain DQ220713 with anterior (a) and posterior flagellum (p) (orientation of flagella: comp. Fig. **3.1**); arrowheads marking the acronomatic part of the anterior flagellum. Scale bars in **3.2** & **3.3** = 5 μm, in **3.4–3.6** = 2 μm.

the cell glides smoothly forward, along its fully extended posterior flagellum. The non-acronomatic posterior flagellum trails underneath the cell body and its length shows significant differences ($p \leqslant 0.001$; $z = -3.311$; U-test) between both compared strains (DQ220712 and DQ220713; Fig. 3.7).

TEM photographs (Figs. 3.8 & 3.9) show that cells from both *Caecitellus* strains follow in general a similar basic structural plan. From the 2 basal bodies, 3 microtubular roots originate. These form 1 compact structured root (R3) and 2 less complex roots (R1 and R4) (Figs. 3.8 & 3.9) (nomenclature according to O'Kelly and Nerad, 1998).

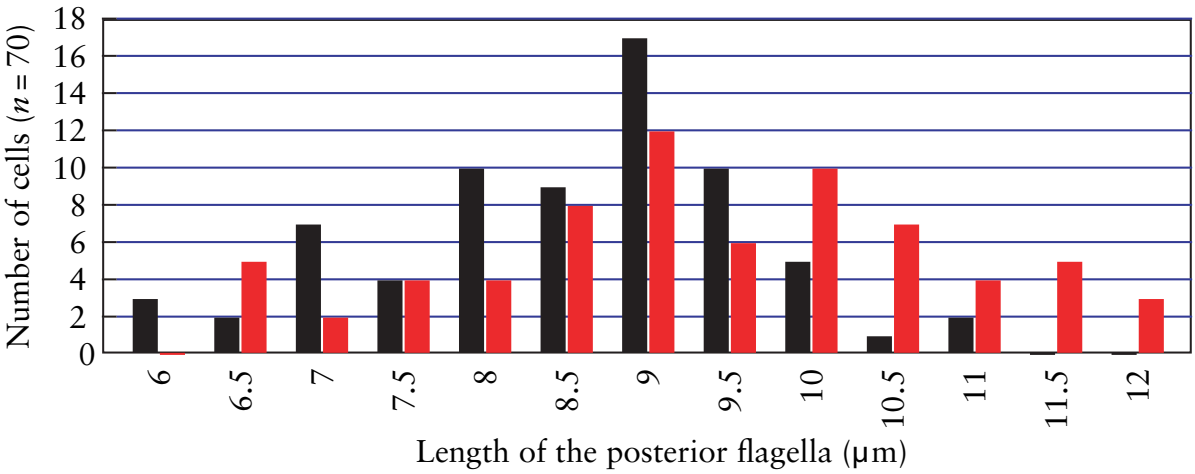
The cells are uninucleate and contain mitochondria with tubular cristae. One mitochondrion (Fig. 3.10) is always located close to the nucleus, at its ventral side and in association with the compact root (R3) at the right side of the cell, next to the so called electron lucent bodies (Fig. 3.10). There is only 1 dictyosome per cell, which is located close to the nucleus and dorsal to the flagellar basal bodies (Fig. 3.11).

There are differences between the examined strains. The glycocalyx of the DQ220712 strain appears as a relatively thick electron dense layer (Figs. 3.8 & 3.10) compared to the glycocalyx of the DQ220713 strain, which is hardly to recognise (Figs. 3.9 & 3.11).

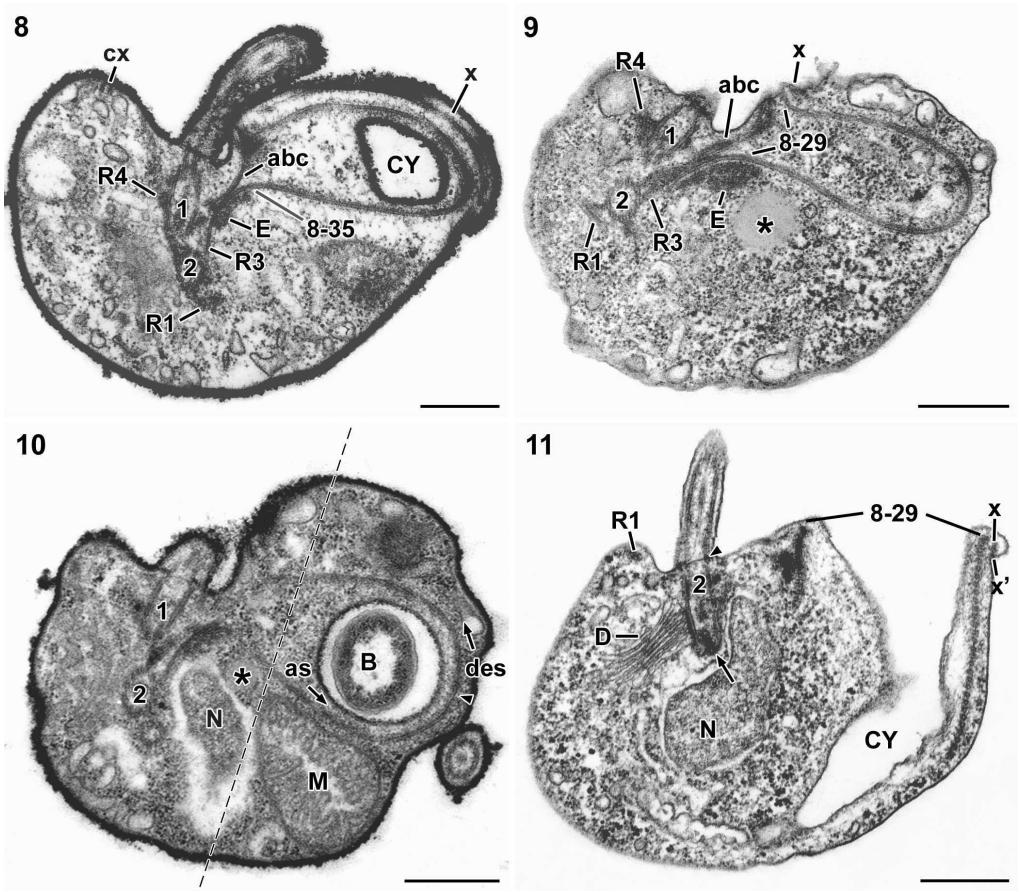
The Flagellar Apparatus

The kinetid contains 2 basal bodies (bb1 and bb2), 1 broad and complex (R3) and 2 simple microtubular roots (R1 and R4) (Figs. 3.8 & 3.9), a striated band (Figs. 3.15–3.17, SB) and a connecting fibre (Fig. 3.18, CF).

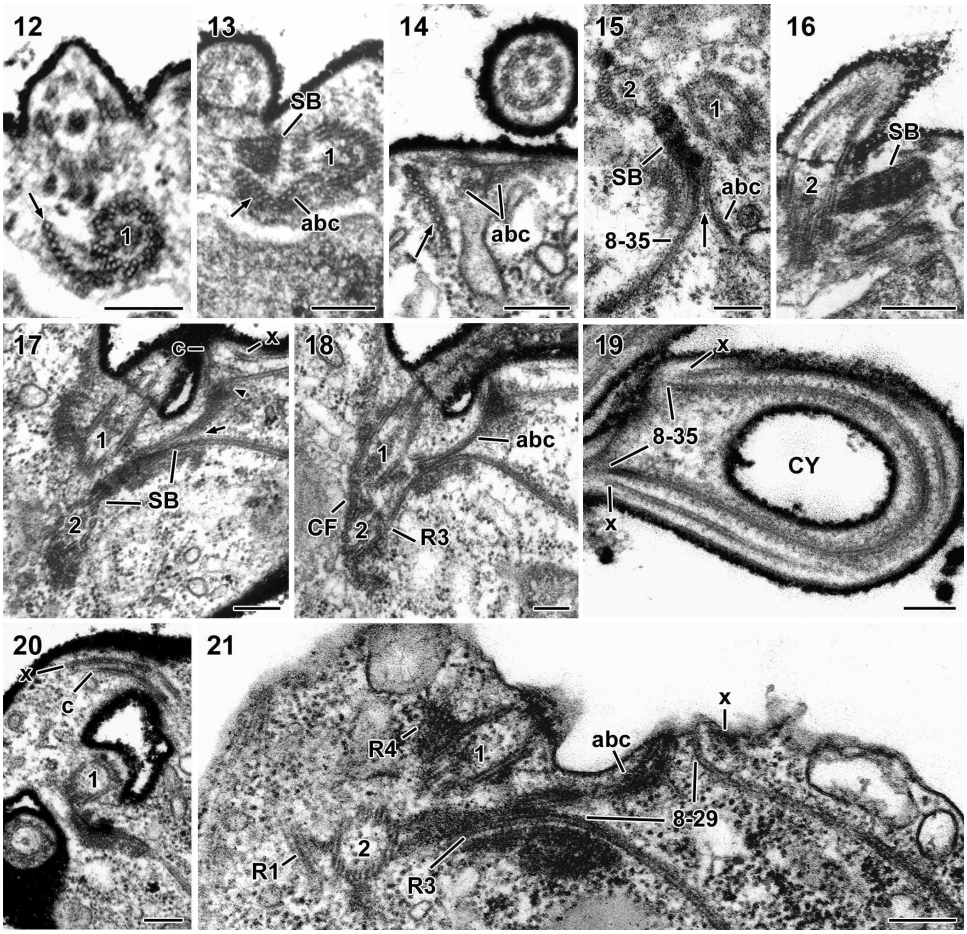
The 2 basal bodies are connected by CF and with R3 by SB. SB leads from the right hand side of bb2 as far away as the point of separation of R3 (Figs 3.16 & 3.17). It runs



Figures 3.7: The diagram shows significant ($p \leqslant 0.001$; $z = -3.311$; U-test) differences in the length of the posterior flagellum comparing 70 cells of *C.* cf. *parvulus* strain DQ220712 (red) from the deep sea with 70 cells of *C.* cf. *parvulus* strain DQ220713 (**black**) from the surface water of the South Atlantic.



Figures 3.8–3.11: Transmission electron micrographs showing the general cellular organisation of the 2 examined *Caecitellus* strains [DQ220712, deep sea (Figs. **3.8** & **3.10**) and DQ220713, surface water (Figs. **3.9** & **3.11**)]. Figures **3.8** & **3.9** Horizontal sections, viewed from anterior/dorsal showing microtubular root 1 (R1) and root 3 (R3) originating from basal body of anterior flagellum (2). R3 splitting into a short loop (abc), a large loop (8–35 resp. 8–29) and a single microtubule (x). Electron-dense material (E) at the separation point of abc loop and 8–35 loop, x-microtubule leading at the right side of the cell around the cytostome (CY). Microtubular root 4 (R4) originating at basal body of posterior flagellum (1). **3.8** Microtubules c and x at the anterior/left side of the cell. **3.9** Electron-lucent body (*) in close vicinity to ascending part of 8–29-loop. **3.10** Mitochondrion (M) with tubular cristae close to the nucleus (N) ventrally next to ascending part (as) of large loop of R3 and to the electron lucent bodies (*). After turning point (arrowhead) large loop turning left, its descending part (des) passing along ventral side of the cell. Bacterium (B) inside oral apparatus. Dotted line = approximate section plane of Figs. **3.20–3.24**. **3.11** Longitudinal section of basal body 2 with electron dense material in its proximal end (arrow) and with basal plate at level of the plasmalemma (arrowhead). Dictyosom (D) close to the left of basal body 2 and the nucleus (N). Prominent cytophaynx (CP) surrounded by a basket of 8–29 microtubules. In the lip microtubule x′ underneath the microtubule x. Root 1 (R1) directly underneath the anterior surface at the left side of the cell. GK = glycocalyx. Scale bars = 0.5 μm.



Figures 3.12–3.21: Kinetid of a *Caecitellus* cell (strain DQ220712, deep sea). **3.12** Cross section of the proximal end of basal body 1 (1) and R3 (arrow). **3.13** Cross section of L-shaped part of R3, basal body 1 and striated band (SB) at a point slightly distal to that of Fig. **3.10**. R3 depicted as L-shaped structure with typical 8+3 pattern, showing the start of separation of abc and 8 subunit (arrow) of R3. **3.14** Cross section of 8 subunit (arrow) and abc microtubules at a level distal to R3-separation. **3.15** Cross section of basal body 2 and oblique section of basal body 1, showing position of striated band (SB) and separation of R3 in abc subunit and 8–35-loop (arrow). **3.16** Oblique section of basal body 2 illustrating its association with striated band (arrow). **3.17–3.19** Consecutive section of the connecting structures of basal bodies 1 and 2, i. e., i) striated band (SB) and connecting fibre (CF), ii) proximal end of R3 with its point of separation (arrow) as well as junction of the descending 8–35 loop, iii) abc loop (arrowhead) , iiii) continuation of microtubules c and x at left side of the cell. **3.19** Longitudinal section of microtubule x and 8–35 loop leading around the cytostome (CY). **3.20** Distal end of R3 consisting of microtubules c and x leading around the insertion of the posterior flagellum (1). **3.21** Kinetid of *Caecitellus* cell strain DQ220713 (surface water), section through the dorsal anterior side of the cell, showing i) basal bodies 1 and 2, ii) the origin of microtubular roots R1, R3 and R4, iii) parts of the subunits of R3 (8-29, abc, x). Scale bars in **3.12–3.21** = 0.2 μm.

slightly anterior to R3 (Fig. 3.13) and has a connection to bb1. CF extends between the bases of the 2 basal bodies from the left hand side of bb2 towards the base of bb1 (Fig. 3.18).

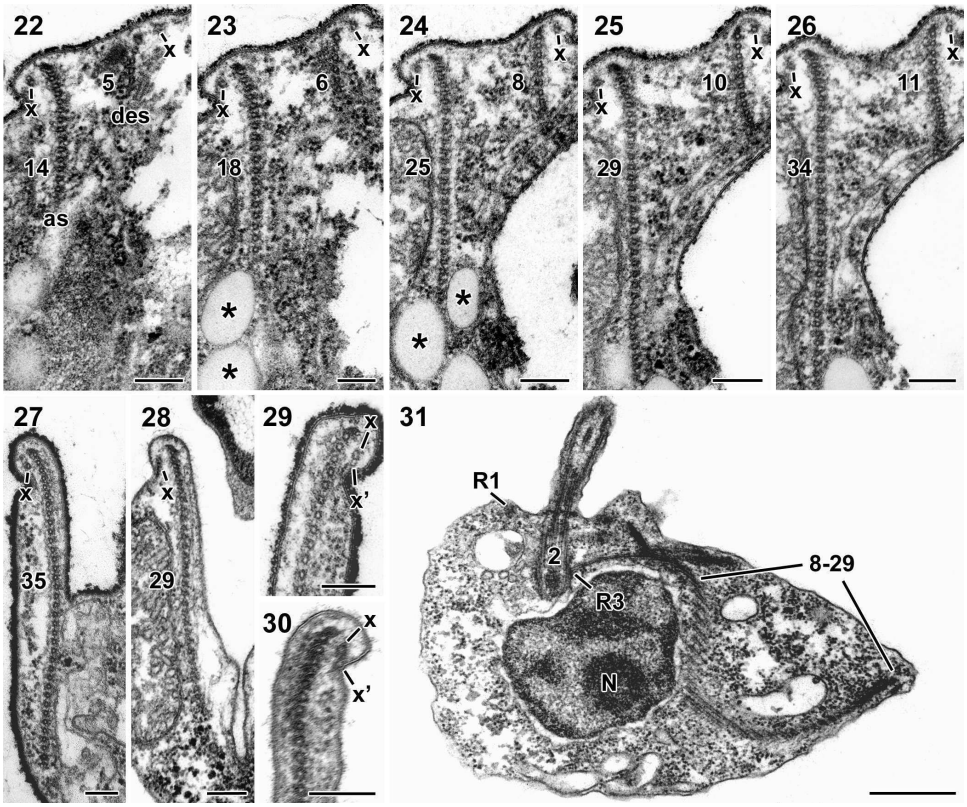
The basal bodies of the posterior (1) and anterior (2) flagellum are approximately 0.5 μm long (DQ220713) and orientated to each other in an L-shaped manner. Their longitudinal axes do not run coplanar, but are approx. 0.15 μm (equally length of CF) shifted to each other and slightly laterally tilted. Electron-dense material is located in the proximal lumen of the basal bodies (Fig. 3.11, arrow). Cross sections of this region show clearly the 9×3+0 structure (Fig. 3.12). There is a basal axonemal plate at the level of the plasmalemma (Fig. 3.11, arrowhead).

R1 composed of 2 microtubules originates in an electron-dense material on the right side of bb2 (Figs. 3.8 & 3.9) at its midregion and extends to the dorsal surface to the left side of the cell (Fig. 3.11). R3 consists of 3 subunits: The abc subunit, the 8–35 subunit (in strain DQ220713, respectively named 8–29 subunit in strain DQ220712) and the x subunit (Figs. 3.8 & 3.9).

At the origin of R3, which lies at the ventral side of the proximal end of bb2, the root consists initially of 8 microtubules (Fig. 3.12). After a short distance, 3 more microtubules are added, which appear in cross section as L-shaped structure with a typical 8+3 pattern (Fig. 3.13). The 3 added microtubules are also known as the abc subunit of R3. It separates from the root (Figs. 3.14 & 3.15) and turns slightly left forming a tight loop around the posterior flagellar insertion (Figs. 3.18 & 3.20). The broadest subunit of R3, initially consisting of 8 microtubules, increases in number up to 35 (Fig. 3.27) and passes to the right side of the ventral region forming a loop that supports the peristome (Fig. 3.19). It then passes left and back to make contact with the abc subunit (Figs. 3.8 & 3.9). At the point of separation, R3 is associated with electron-dense material subtending the 8 microtubule subunit (Fig. 3.21, E). In the ascending root (Fig. 3.10), which is the broader subunit of R3, the number of microtubules increases. In the deep sea strain (DQ220712), 35 microtubules (Fig. 3.27), and in the surface strain (DQ220713), as many as 29 microtubules (Fig. 3.11), have been detected. The highest number of microtubules is reached just before the turning point of the loop. Figures 3.22–3.26 are serial sections through the oral apparatus. The section plane of these pictures is indicated in figure 3.10 by a dotted line. The microtubules of the ascending side of the loop increase in number compared to the descending side of the loop (Figs. 3.22–3.26).

These extra microtubules have no obvious connection to the basal bodies, whereas the junction of the large-loop and the abc loop is visible in the figures 3.17 and 3.19. Most microtubules of the descending root terminate before both loops join. In figure 3.22, there are only 5 microtubules left in the descending root.

Parallel to the large-loop runs an individual microtubule, which is called x (Figs. 3.11, 3.19, 3.22–3.30). This microtubule has its origin close to the separation of R3 and extends to the outside of the loop in the lip at approximately the same level as the second microtubule from the 8–35 loop. In the area of the turning point of the x-microtubule one additional microtubule is visible directly underneath it (Figs. 3.29 & 3.30).



Figures 3.22–3.31: Microtubular structure of the feeding basket from a *Caecitellus* cell (strain DQ220712) in serial sections (section plane = dotted line in **3.10**) showing i) increasing number of microtubules in ascending (as) and descending (des) part of 8–35 subunit of R3, ii) location of electron lucent bodies (*), iii) position of microtubule x. **3.27** Ascending root with max. 35 microtubules (strain DQ220712) and in **3.28** with max. 29 microtubules in strain DQ220713. **3.29** (DQ220712) and **3.30** (DQ220713) show the microtubule x′ in the lip directly underneath microtubule x in the area of the turning point of the loop. **3.31** Cell of strain DQ220713 in dorsal view illustrating basket structure of 8–29 loop (ascending subunit), origin of root 3 (R3) at basal body 2 anteriorly to the nucleolus (N) and location of R1 at the left anterior side of the cell. Scale bars in **3.22–3.26**, **3.29**, **3.30** = 0.2 μm, in **3.27**, **3.28**, **3.31** = 0.5 μm.

The end of R3 is made of the microtubule c and x, which combine the small and the large loop at the left side of the cell, and run around the insertion region of the posterior flagella towards the dorsal apical cell side (Figs. 3.17 & 3.19).

R4 consists of 2 microtubules, which arise from the basal body of the anterior flagellum (Figs 3.8, 3.7 & 3.21). It leads from the dorsally oriented part of the correspondent basal body to the left ventral side of the cell. It terminates near the end part of R3.

Summarising, the strains DQ220712 and DQ220713 show significant differences in the length of the posterior flagellum, the appearance of the glycocalyx and the maximal number of microtubules in R3.

Molecular Data

All sequences of *Caecitellus* obtained in this study have nearly the same length as those reported from previous studies (Tab. 3.1). After an initial phylogenetic analysis comprising a broad range of heterokont taxa, the labyrinthulid *Ulkenia profunda* has been chosen to root the tree. These initial analysis supported the placement of *Pseudobodo tremulans* as basal bicosoecid taxon (Karpov et al. 2001), although its placement at the root of the bicosoecids varies depending on the taxa and the numbers of sequences included in phylogenetic analysis. An unstable branching pattern has been observed regarding the bicosoecid *Symbiomonas scintillans*. This taxon has therefore been excluded from phylogenetic analysis. With the exception of *Cafeteria*, all phylogenetic methods recovered the same optimal tree topology with each node supported by high bootstrap support (Fig. 3.32). In ML and MP analysis, *Cafeteria* branches at the root of the *Caecitellus* clade with moderate bootstrap support (ML: 65; MP: 52), in ME analysis at the root of the *Adriamonas*/*Siluania* clade (bootstrap value: 64).

The clade composed by both *Pseudobodo* strains branches first, followed by the clade composed by *Siluania*, *Adriamonas* and *Cafeteria* and finally the clade comprising the different strains of *Caecitellus*. The tree shows a paraphyletic family Siluaniidae (*Siluania*, *Adriamonas* and *Caecitellus*; Karpov 2001) and Cafeteriidae (*Cafeteria* and

Table 3.2: *P*-distances of *Caecitellus* in percent (pairwise-deletion option set). GenBANK accession numbers are given. Strains sequenced in this study are in **bold**.

	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
(1) AF174368										
(2) AF174367	0.00									
(3) DQ220715	4.55	4.44								
(4) DQ220716	4.60	4.49	0.00							
(5) DQ220713	4.39	4.39	0.00	0.00						
(6) AY827847	4.54	4.55	0.00	0.00	0.00					
(7) AY827848	4.65	4.66	0.12	0.12	0.13	0.12				
(8) DQ220717	4.48	4.49	0.06	0.12	0.00	0.06	0.12			
(9) DQ220714	4.38	4.39	0.00	0.00	0.00	0.00	0.12	0.00		
(10) DQ220712	5.96	5.96	5.28	5.28	5.30	5.28	5.50	5.28	5.28	
(11) DQ230538	5.85	5.86	5.55	5.60	5.24	5.54	5.46	5.20	5.17	0.00

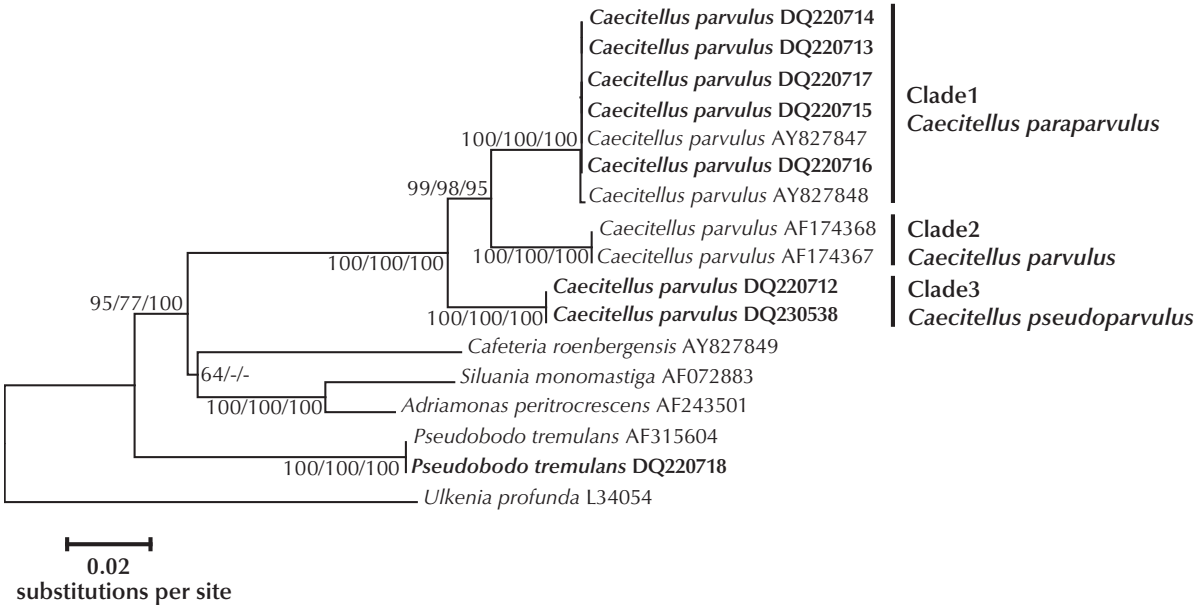


Figure 3.32: Rooted minimum evolution bootstrap consensus tree of the order Bicosoecida (Grassé), Karpov 1998, using 1533 positions (Nucleotide substitution model: LogDet; complete-deletion option set). The tree was rooted using *Ulkenia profunda* as outgroup. Numbers at the nodes are bootstrap support percentages from 250 replicates (ME, left), respectively 100 replicates (ML, middle; MP, right). Strain identifiers refer to GenBANK accession numbers. Strains sequenced in this study are in **bold**.

Pseudobodo; Moestrup 1995), but discrepancies between morphological and molecular data concerning this families are well known and have already been addressed in detail by Karpov et al. (2001).

In all trees obtained, *Caecitellus* forms a monophyletic group with 3 distinct clades and with very high bootstrap support values (Fig. 3.32). The first clade (Clade1—*Caecitellus paraparvulus*) is composed by the strain isolated in 1981 by P. G. Davis, and which has been studied by O'Kelly and Nerad (1998; DQ230538), and the strain isolated in 2000 from deep-sea sediments of the Angola Abyssal Plain and which ultrastructure is subject to this study (DQ220712). The second clade (Clade2—*Caecitellus parvulus*) is composed of both strains sequenced by Atkins et al. (2000) and isolated in 1995 from mussel beds of deep-sea hydrothermal vents of the Eastern Pacific Rise (AF174367), respectively 1996 from New Bedford Harbor, Massachusetts (AF174368). The third clade (Clade3—*Caecitellus pseudoparvulus*) is composed of strains, which have all been isolated in 2000 from sediments of the Angola Abyssal Plain, with the exception of one strain from surface water of the South Atlantic Ocean, which ultrastructure is also subject to this study (DQ220713).

Uncorrected distances (*p*-distances) have been calculated for all strains of *Caecitellus* (Tab. 3.2) and show very high sequence divergences within this group, as well as for both strains of *Pseudobodo* (0.20 %). Mean *p*-distances and their standard deviations have been calculated between the 3 major clades of *Caecitellus*. as well as within these clades. Mean distances within all 3 clades are equally low with minimal variances (Clade1: 0.00 %; Clade2: 0.00 %; Clade3: 0.05 ± 0.03 %). On the other hand, very high mean distances, with at the same time low variances, can be observed in all 3 cases between these clades (Clade1/Clade2: 5.91 ± 0.54 %; Clade1/Clade3: 5.35 ± 0.52 %; Clade2/Clade3: 4.50 ± 0.50 %).

Discussion

Morphological Data

At the level of light-microscopy there are no differences visible between the investigated *Caecitellus* cf. *parvulus* strains, i. e., strain DQ220712 from the deep sea and strain DQ220713 from the surface water of the South Atlantic. Their cell shape and way of movement seems to be in conformity with almost all former light microscopic descriptions (e. g. Patterson et al. 1993, Tong 1997a, O'Kelly & Nerad 1998, Lee & Patterson 2000, Al-Qassab et al. 2002, Lee et al. 2003). An additional thread trailing from the outer margin of the mouth, only noted from Tong (1997b) and Tong et al. (1998) was not seen in the present study. However, biometric analysis showed significant differences in the length of the posterior flagella of both studied strains (*C.* cf. *parvulus* strain DQ220712 and DQ220713). But the average lengths of the posterior flagella in both strains are within the size range reported in the literature (e. g. O'Kelly & Nerad 1998, Atkins et al. 2000, Al-Qassab et al. 2002, Lee et al. 2003).

The ultrastructural comparison of *Caecitellus* cf. *parvulus* strain DQ220712 and strain DQ220713 shows that in general both strains follow a similar basic structural plan, but there are differences in the appearance of the glycocalyx and in the maximal number of microtubules in the flagellar root 3 (R3) (Tab. 3.3, Figs. 3.33 & 3.34).

Only in the strain *C.* cf. *parvulus* DQ220712, the glycocalyx is visible as a relatively thick electron dense layer. It is hardly to recognise at all in the strain *C.* cf. *parvulus* DQ220713 and in all published TEM photographs of *Caecitellus parvulus* (O'Kelly & Nerad 1998, Patterson et al. 1993). Differences in the appearance of the glycocalyx are useful tools to distinguish between the amoeba species *Vanella* and *Platyamoeba* at the ultrastructural level. But molecular studies revealed that the glycocalyx appearances are not a reliable phylogenetic marker to distinguish both species (Page & Blakey 1979, Sims et al. 2002).

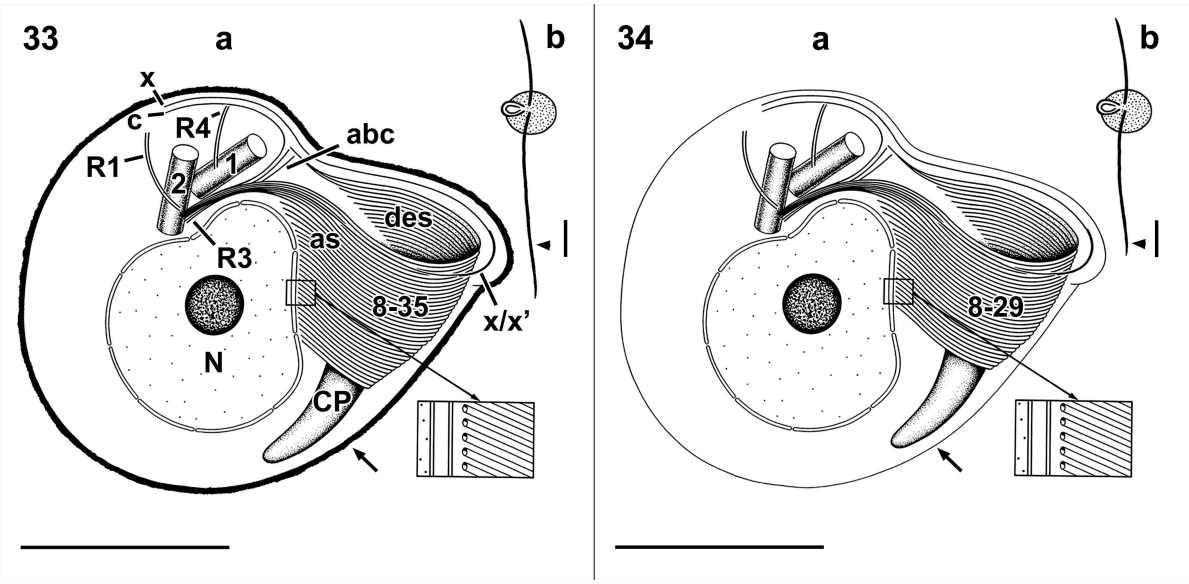
Species-specific differences in the composition and appearance of the glycocalyx do exist during differentiation of parasitic protozoa, like *Blastocystis hominis*, *Giardia intestinalis* and *Trypanosoma brucei* (Zaman and Howe 1997, Zitzmann et al. 2000, Ali & Hill 2003).

The kinetids of both ultrastructural investigated *C.* cf. *parvulus* strains also differ from the kinetid of the described *Caecitellus parvulus* in the maximal number of microtubules found in R3, which build the cytoskeletal fundament of the feeding basket. O'Kelly and Nerad (1998) counted for *C. parvulus* (strain ATCC50712 from the Pacific Ocean) a maximum of approximately 24 microtubules. With 11 microtubules more strain *C.* cf. *parvulus* DQ220712 from the South Atlantic deep sea has a much larger

Table 3.3: Summary of differences between the species of the *Caecitellus* complex.

	***Caecitellus* complex**				
	Caecitellus parvulus			***Caecitellus pseudoparvulus***	***Caecitellus paraparvulus***
	Patterson et al. 1993	O'Kelly & Nerad 1998	Atkins et al. 2000		
cell length	4–7 µm	3–7 µm	3–10 µm	2–4 µm	2.5–4.5 µm
anterior flagella comp. to cell length	?	1.5–2.5 ×	1 ×	1–2 ×	1–2 ×
posterior flagella comp. to cell length	?	2.5–3.5 ×	3–4 ×	2.5–4.5 ×	2–4.5 ×
glycocalyx	thin	thin	?	thick	thin
R1	?	2 mt	?	2 mt	2 mt
R3 (proximal)	?	8+3	?	8+3	8+3
R3 (maximal)	?	24 mt	?	35 mt	29 mt
x -mt (R3)	?	+	?	+	+
x′-mt (R3)	?	—	?	+	+
electron-... bodies associated with R3	dense	lucent	?	lucent	lucent
R4	?	2 mt	?	2 mt	2 mt
genetical distance to *C. parvulus*	?	?		5.91 %	4.5 %
genetical distance to *C. pseudoparvulus*	?	?	5.91 %		5.35 %
genetical distance to *C. paraparvulus*	?	?	4.5 %	5.35 %	

? = not reported; R1 = root 1; R2 = root 2; R3 = root 3; mt = microtubule.



Figures 3.33 & 3.34: Reconstruction of the position of the basal bodies 1 and 2, the paths of the flagellar roots R1, R3 and R4 in relation to the contour of the cell, the nucleus (N) and the cytophaynx (CY) in *Caecitellus* cf. *parvulus*, strain DQ220712 (**3.33a**) and strain DQ220713 (**3.34a**), seen from dorsal. Feeding basket built of 8–35 (**3.33a**) or 8–29 (**3.34a**) microtubules with ascending (as) and descending (des) parts. Separation of R3 into subunits abc, 8′, x. Turning point with additional microtubule x′ directly underneath x. Microtubules c and x representing end of R3 at left/ventral side of the cell. Inset: Microtubules of feeding basket without intimate contact with the nuclear envelop (NE). Differences in the appearance of the glycocalyx of both strains are indicated (arrow). Figures **3.33b** and **3.34b** shows drawings of whole cells of *C.* cf. *parvulus*, strain DQ220712 (**3.33b**) and strain DQ220713 (**3.34d**) seen from ventral, arrowheads highlight the differences in the length of the posterior flagella.

feeding basket. Nevertheless the feeding basket of *C.* cf. *parvulus* strains DQ220713 (from the South Atlantic surface water) with its 29 microtubules is smaller then the basket of *C.* cf. *parvulus* strain DQ220712, but it includes still more microtubules than described for *C. parvulus* (O'Kelly & Nerad 1998).

The differences of the glycocalyx and the feeding basket could be indicative for a different ecological niche and geographic distribution, but the molecular data show that the 3 resulting morphological and genetically distinctive clades include strains from different habitats and locations. Whether the differences in the feeding basket size are possibly coupled with differences in the size of the prey (bacteria), are so far not known.

Molecular Data

The high level of genetic divergence within the morphospecies *Caecitellus parvulus* and the high bootstrap support for the 3 clades suggest that this species complex represents an assemblage of microscopically similar morphotypes united by morphological traits visible on the level of ligth-microscopy: one trailing flagellum, one stiffly and slowly moving anterior flagellum, flattened and often triangular in profile (Patterson et al. 1993, O'Kelly & Nerad 1998, Lee et al. 2000). There is a complete phylogenetic separation between all 3 clades, which form stable and highly separated, coherent phylogenetic clusters with all methods used. The topology of the *Caecitellus*-cluster suggest that the morphospecies *C. parvulus* is no longer maintainable and that it will need to be divided into 3 different species. They do not cluster together on their geographical origins or habitat: strains from the South Atlantic Ocean and the North Atlantic Ocean, as well as strains from the Eastern Pacific Ocean and the North Atlantic Ocean are identic. Besides of the molecular data, the only way to reliably distinguish at least 2 (Clade 1 and Clade 3) of the 3 lineages are the ultrastructural data presented in this study, as well as for one lineage (Clade 2) the ultrastructural data from O'Kelly and Nerad (1998).

Conclusions

The ultrastructural distinction and the large genetic differences between the 3 clades of the morphspecies *Caecitellus parvulus* as well as the high degree of the genetic identity within each genotype demonstrate the existence of at least 3 species within a *Caecitellus* complex. Changes in cell morphology of protist morphospecies whose morphology seems to be driven by high selection into adaptive peaks (Nanney 1982), is assumed to be the result of speciation events and should thus reflect disinct species (Andersen 1998, Finlay 2004).

Recent molecular studies indicate that several cryptic species might exist among protists (e. g. Nanny et al. 1998, Darling et al. 2004, Scheckenbach et al. 2005). At least in some cases, detailed comparisons of morphological and non-morphological features showed that also pseudo-cryptic species exist and slight morphological differences may separate species (Huber et al. 1997, Darling et al. 1999, de Vargas et al. 1999, Sáez & Lozano 2005). Therefore the results of the present study indicate a new description of 2 new *Caecitellus* species. One called *Caecitellus paraparvulus* includes the strains of Clade 1 of this study, the other new species *Caecitellus pseudoparvulus* in-

cludes both strains of Clade 3 (Fig. 3.32). O'Kelly and Nerad (1998) show only light microscopy photographs of the now newly termed strain *Caecitellus pseudoparvulus* DQ230538 (ATCC50091) isolated from the Sargasso Sea. For the ultrastructural description of *Caecitellus parvulus*, they use the strain ATCC50512, which was isolated from the North Pacific Ocean. Unfortunately, the strain *C. parvulus* ATCC50512 is not available anymore, e. g., from the "American Type Culture Collection", were it was deposited (O'Kelly & Nerad 1998). Therefore at present it does not seem to be possible to investigate the genotype of this strain. Clade 3 includes 2 strains of *C. parvulus* which were sequenced and light microscopically described by Atkins et al. (2000).

As pointed out by de Vargas et al. (1999) for planktonic foraminifers, our results, including the results of Scheckenbach et al. (2005), indicate for *Caecitellus parvulus* as a case study of heterotrophic nanoflagellates, that the world-wide species diversity might be greatly underestimated if a morpho-species concept is applied. Different strains of the 3 species of the genus *Caecitellus* were found in different locations or habitats, consequently it seems that there is no evidence for endemism of the respective *Caecitellus* species, but special micro-environmental and behavioural conditions might exist.

Finally, our results show the potential of combined DNA and ultrastructural analyses for detection of species complexes within morphospecies of heterotrophic flagellates.

Taxonomic Diagnosis

Genus *Caecitellus* (Patterson et al. 1993) – *Caecitellus* compl. nov. (Table 3.2) Distinguishably at the level of light microscopy among gliding flagellates by a conspicuous ventral mouth, the orientation of the 2 flagella and the beat pattern of the anterior flagellum (Al-Qassab et al. 2002). Cell sizes from 2–10 μm have been reported. The small heterotrophic nanoflagellates have somewhat rounded or triangular profiles and feed on attached bacteria. The acronematic anterior flagellum inserts apically, is about 1–2.5 times the cell length and beats anteriorly and stiffly. The measurements for the length of the posterior non-acronematic trailing flagella range from 2 times till 4.5 times the cell length (e. g. Griessmann 1913, Larsen & Patterson 1990, Patterson et al. 1993, O'Kelly & Nerad 1998, Tong et al. 1998, Lee & Patterson 2000, Al-Qassab et al. 2002, Lee et al. 2003, present study).

***Caecitellus parvulus* (Basionym: *Bodo parvulus,* Griessmann 1913) Patterson et al. (1993)** For detailed ultrastructural description see O'Kelly and Nerad (1998).

***Caecitellus paraparvulus* spec. nov.** Likewise the first described *Caecitellus* species *C. paraparvulus* has basically the same ultrastructure as described by O'Kelly and Nerad (1998) for *C. parvulus*. The maximal number of microtubules of the large loop of R3 is different. With approximately 29 microtubules, *C. paraparvulus* has 5 microtubules more than *C. parvulus* and 6 microtubules less than *C. pseudoparvulus* in its feeding basked. As described for *C. pseudoparvulus*, there is a x′-microtubule underneath the x-microtubule. The glycocalyx of *C. paraparvulus* is thin or hardly to recognise at all. Compared to *C. pseudoparvulus*, this species has in average a shorter posterior flagellum.

***Caecitellus pseudoparvulus* spec. nov.** The kinetid of *Caecitellus pseudoparvulus* is basically similar to the kinetid of *C. parvulus* described by O'Kelly and Nerad (1998). The maximal number of microtubules of the large loop of R3 is different. 35 microtubules were counted in the cytostome for *C. pseudoparvulus*. An additional microtubule, x′, is located in the area of the turning point of the large loop directly underneath microtubule x, and firstly described in this study for *C. pseudoparvulus* and *C. paraparvulus*. Within the *Caecitellus*-complex only *C. pseudoparvulus* shows a relatively thick electron dense glycocalyx. *Caecitellus pseudoparvulus* has in average a longer posterior flagellum than *Caecitellus paraparvulus*.

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Part IV.

Where are all the protists?

The high extent of cryptic species diversity found within morphologically similar species (Sáez & Lozano 2005)—from the ciliate *Paramecium aurelia* (Sonneborn 1939, Coleman 2005) to the butterfly *Astraptes fulgerator* (Hebert et al. 2004)—has led to the assumption that morphologically defined species (morphospecies; Cain 1954) of eukaryotic microbes (protists) are just a facade behind which lurk a high number of morphologically indistinguishable—cryptic—species (Cairns 1993). Since different species should diverge genetically with time, we applied DNA barcoding based on 1,514 complete sequences of nuclear small subunit ribosomal DNA (SSU rDNA) of 386 morphospecies retrieved from GenBANK with a cut-off value of 0.50 % genetic divergence between distinct operational taxonomic units (OTUs), to investigate the extent of cryptic diversity within protist morphospecies. Here we show that the genetic diversity within morphospecies is generally very high with 2.56 % mean intraspecific genetic distance—equal to the genetic distance between human and, e. g., the North American opossum (2.53 %)—and that one half of the morphospecies contain cryptic species leading to a gross underestimation of protist species diversity with 1 order of magnitude more species estimated than present nominal species. Morphology obviously often masks evolution. Integrative taxonomy is needed in order to shed light on all the species hiding behind the facade of a morphospecies (Tautz et al. 2002, Blaxter 2004, Dayrat 2005).

Protist diversity is still heavily disputed. Basically, there are 2 opposing views: there are many species, as suggested by the log-normal species-body-length relationship, with the a priori expectation that there should be more small than large taxa; or there are few species, as suggested by the actual number of nominal species (May 1988). The actual number of nominal species of protists is low (10^4), and their estimated number lies only in the range of 10^5 species, in contrast to the other microbial group—the prokaryotes—where the number of named bacterial species is in fact equally low (9,406; "www.bacterio.cict.fr"), but their estimated number goes into the millions and even billions (Dykhuizen 1998, Torsvik et al. 2002). Since the estimated "grand total" of all eukaryotic species is $6.8 \cdot 10^6$ (May 1997), the most abundant, evolutionary oldest and phylogenetically most diverse eukaryotes, key players in most ecosystems worldwide, would count for only a small portion of Earth's eukaryotic diversity. Knowledge of the number of protist species is not trivial but a fundamental issue concerning Earth's biodiversity.

The main difficulties in assessing protist diversity comes from the fact that protist species classification is based on morphology and that similarity of form is not equivalent to genetic, biological or ecological identity (Schlegel & Meisterfeld 2003). Since appearance can be misleading, morphology is often inadequate in defining the boundaries of the taxon "species" and thus results in a lumping of species. Speciation processes might be rather coupled to other phenotypic aspects than to morphology, which represents in many cases adaptive peaks and thus masks evolution. Given the relationship between phenotypic evolution within nominal species and genetic divergence demonstrated by several studies (Darling et al. 2000, Norris & de Vargas 2000, Coleman 2001, Sáez et al. 2003), high genetic distances between slowly evolving housekeeping genes, such as the SSU, should reflect speciation events, rather than simply the accu-

mulation of selectively neutral mutations as suggested by some authors (Finlay 2002, Fenchel 2003). DNA barcoding using the microbial "gold standard" SSU (Charlebois et al. 2003) seems to be an auspicious, pragmatic, operational and universally applicable approach in identifying microbial taxa cryptic to morphological analysis (Blaxter 2003). Although an OTU definition is a priori arbitrary and controversial and might vary fom species to species, it serves as a useful guideline for analysis and communication (Hughes et al. 2001, Kemp & Aller 2004). Since speciation is a continuous process and can occur at any time along the course of evolutionary lineages, all values for OTU delineation must be taken as upper limit, where speciation events should have taken place.

In total 1,514 sequences of 386 protist morphospecies (S) have been retrieved from GenBANK and a value of 0.50 % p-distance has been taken for OTU delineation. Plotting the number of intraspecific comparisons against the respective genetic distance, results in an exponential distribution with a distinct peak at 0.00 % p-distance (mode), with null skewness, and heavy tails (kurtosis = 13.03), indicating that high intraspecific distances are present (Fig. 4.1.A). This is reflected by a very high mean intraspecific genetic p-distance and standard deviation (2.56 ± 4.30 %) and—since not normally distributed—by the more appropriate high median value (0.94 %, $Q_1 = 0.20$ %, $Q_3 = 2.26$ %; Fig. 4.1.A). A table summarising the basic statistics for every morphospecies is available in Supplementary Information. These high intraspecific distances strongly suggest that a high number of cryptic species should be present within the morphospecies analysed. Indeed, whereas 38% of all morphospecies contain identical sequences, only 17.35 % are genetically identical (0.00 % p-distance), and half of the morphospecies (50.40 %) contain cryptic species, according to the OTU definition given. The observed number of species (S_{obs}), within the morphospecies analysed, is 727 (OTU $\leqslant$ 0.50% p-distance; $S_{obs}^{0.50\,\%}$; Fig. 4.1.B). Since 0.50% divergence is the upper limit for OTU delineation, the actual number of species, within the morphospecies analysed, lies between $S_{obs}^{0.50\,\%}$ and 1,205—the number of species for an OTU definition of 0.00 % p-distance ($S_{obs}^{0.00\,\%}$).

Using the species richness estimator Chao1, one can extrapolate from SSU sequence dissimilarity, how many species might be present in total (Hughes et al. 2001, Kemp & Aller 2004). The estimated total species number, within the morphospecies analysed, (S_{Chao1}) lies between 1,149 (OTU $\leqslant$ 0.50 %p-distance; $S_{Chao1}^{0.50\,\%}$; Fig. 4.1.B) and 6,869 (OTU = 0.00 % p-distance; $S_{Chao1}^{0.00\,\%}$; Fig. 4.1.C). The protist diversity is presently 1.9–3.1 $\times$ ($S_{obs}^{0.50\,\%}/S$–$S_{obs}^{0.00\,\%}/S$), and estimated to be 3.0–17.9 $\times$ ($S_{Chao1}^{0.50\,\%}/S$–$S_{Chao1}^{0.00\,\%}/S$) higher, than the actual number of nominal species as a result of cryptic diversity. The total number of species should therefore be at least 1.9–17.9 $\times$ ($S_{obs}^{0.50\,\%}/S$–$S_{Chao1}^{0.00\,\%}/S$) higher than the number of morphospecies.

Whereas $S_{Chao1}^{0.50\,\%}$ levels off and can be considered to be a reasonable estimate of the actual species richness, $S_{Chao1}^{0.00\,\%}$ failed to reach saturation. The number of estimated species for OTU = 0.00 % p-distance is therefore an inaccurate richness estimate, although the magnitude should be regarded as likely. The GenBANK is not a systematic sampling effort, but it is the largest available dataset of Earth's protist genetic diversity, and still it is not large enough for reliably estimate the upper value of the total protist species

number ($S_{obs}^{0.00\%}$) present within the 386 morphospecies analysed. Most morphospecies contain only 2 or few more sequences, whereas just very few morphospecies have been sampled exhaustively (Fig. 4.1.D). The extent of intraspecific genetic diversity might therefore increase with further sampling of the present morphospecies.

The results in this letter clearly show that most morphospecies of protists consist most likely of multiple morphologically indistinguishable cryptic or pseudo-cryptic species. Protist species diversity should therefore be considered to be 1 order of magnitude higher due to cryptic diversity. If one bears in mind, that α-taxonomists like Foissner (1999) and geneticist like Nanney and colleagues (1998) have argued for ciliates, that the estimated number of species should be regarded as 10 × higher than it is actually, the total number of protist species is expected to be at least in the range of 10^7 species. Since cryptic species appear to be a general phenomenon, the numbers of protist species may indeed be as large as suggested by Hutchinson's and MacArthur's (1959) log-normal species-body-length relationship. Moreover, since estimates of protist diversity based on SSU sequences probably represent a minimum of the real biological, ecological and genetic diversity.

The morphospecies obviously rather reflects the—humble—morphological diversity of protists rather than valid species. Morphology often seems to represent a fitness of form which is driven by strong selection into adaptive peaks, where it is "frozen and doomed to remain there forever" (Nanney 1982), while other genetical or eco-physiological characters can change. Similar morphology might thus be the result of morphological convergent or parallel adaptive evolution (Simpson 1961), or simply of a common ancestry, and not necessarily the expression of a membership to a distinct species.

We need to know why cryptic species exist and what their nature is. Looking at the high extent of cryptic species and the obvious shortcomings of morphology in reliably identifying species, protistology has to set up a taxonomical framework, integrating morphology, ecology and definitively also genetics, while avoiding the over-emphasising of DNA barcoding leading to the "Land of the One-Eyed King" (Forney et al. 2004), where prokaryotic microbiology is actually trapped, but also the morphology-dominated classical taxonomy, leading to a fuzziness in species definition and consequently to a lumping of protist species under the morphospecies umbrella. Integrative taxonomy will shed light on the extent of intra- and interspecific variation in order to accurately define species boundaries and, hereupon, on all the species hiding behind the facade of a morphospecies (Tautz et al. 2002, Blaxter 2003, Dayrat 2005). This is definitively "no trivial pursuit" (Nanney 2004), and has still to be done for the vast majority of protist model organisms, but has definitively to be carried out, if one will ever get a glance of one of the greatest knowledge gaps in protistology—protist diversity.

Methods

Protist morphospecies with at least 2 available nuclear encoded SSU sequences ($\geqslant 1.5$ kb) have been retrieved from GenBANK in December 2004, excluding groups containing multicellular organisms (Fungi, Metazoa, Rhodophyta and Viridiplantae). Alignments

have been carried out with ClustalX 1.83 (Thompson et al. 1994). Uncorrected *p*-distances and linearised minimum evolution phylogenetic trees have been calculated with MEGA 3.0 (Kumar et al. 2004). OTU delineation for a definition of $\leqslant 0.50$ % *p*-distance has been done by applying a cut-off value of 0.0025 to the linearised trees computed; for a definition of 0.00 % *p*-distance by direct comparison of *p*-distances. Basic statistics have been calculated using GNU awk. Species accumulation curves and richness estimation curves have been calculated with EstimateS 7.50 (Colwell 2005).

Supplementary Information

A table summarising the basic statistics for every morphospecies is available in the Appendix A.

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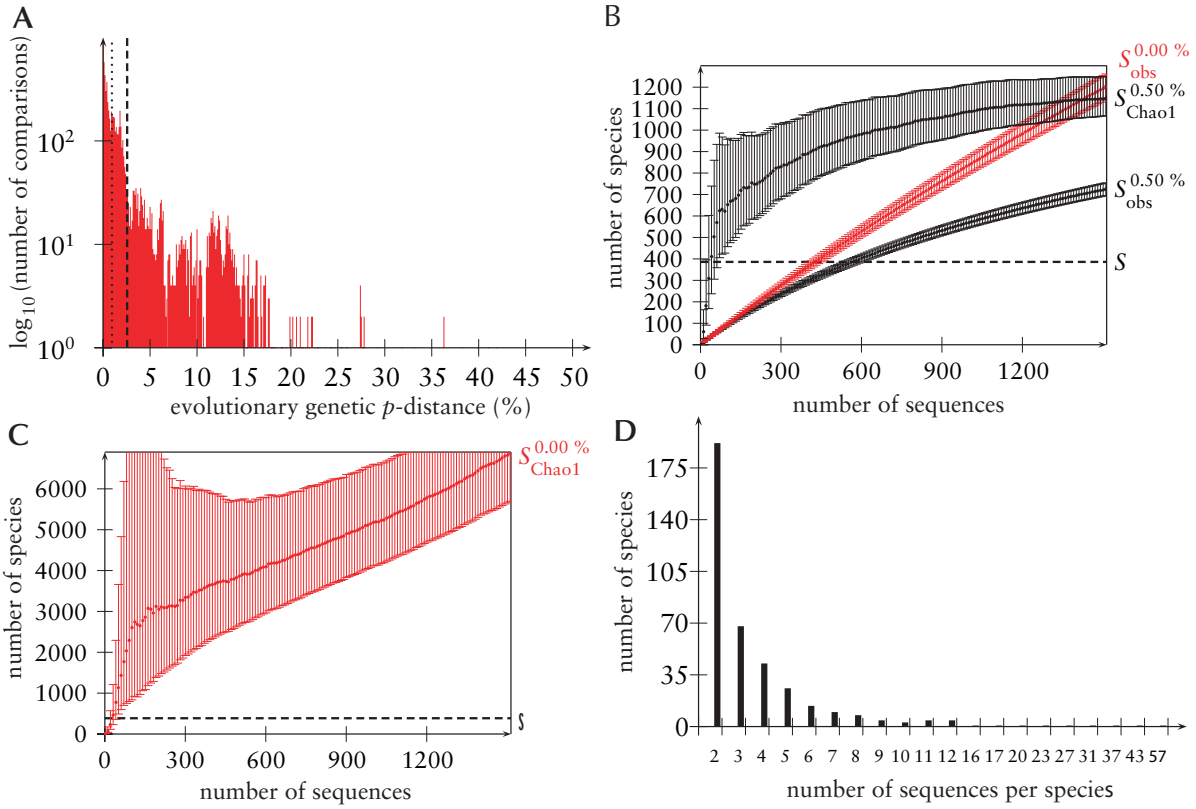

Figure 4.1: **A:** Logarithmic display of the number of intraspecific sequence comparisons for a given genetic *p*-distance (**red**). Mean *p*-distance = 2.56 % (dashed vertical line); median *p*-distance = 0.94 % (dotted vertical line). **B & C:** Accumulation and richness estimation curves (Chao1) for the observed (S_{obs}) and estimated (S_{Chao1}) number of species using an OTU definition of $\leqslant 0.50$ % *p*-distance ($S_{obs}^{0.50\,\%} = 727$; $S_{Chao1}^{0.50\,\%} = 1{,}149$; **black**) and 0.00 % *p*-distance ($S_{obs}^{0.00\,\%} = 1{,}205$; $S_{Chao1}^{0.00\,\%} = 6{,}869$; **red**). Error bars are 95 % confidence intervals. Number of nominal species (S) is 386 (dashed horizontal line). **D:** Number of sequences available per morphospecies against number of morphospecies for groups with at least 1 morphospecies. Protist morphospecies with at least 2 available sequences of nuclear small subunit ribosomal DNA (SSU rDNA $\geqslant 1.5$ kb) have been retrieved from GenBANK excluding groups containing multicellular organisms (Fungi, Metazoa, Rhodophyta and Viridiplantae)—in total 386 nominal species with 1,514 sequences.

Abstract

Protist diversity is still heavily disputed. The fundamental issue results from the fact that there are apparently by far less species than should be a priori expected by Hutchinson's and MacArthur's (1959) log-normal species-body-length relationship (May 1999). Morphology remains the "gold standard" for species identification for more than 200 years, but it has some severe shortcomings, as protists often lack distinguishable morphological traits (Schlegel & Meisterfeld 2003). The high extent of morphologically indistinguishable—cryptic—species, found within nominal species (Sáez & Lozano 2005), has led to the assumption that morphologically defined species (morphospecies; Cain 1954) of protists are just a facade behind which lurk a high number of cryptic species (Cairns 1993).

Since different species diverge genetically with time, the intraspecific genetic distance of the nuclear small subunit ribosomal DNA (SSU rDNA) of some of the world-wide most common heterotrophic flagellated morphospecies (*Amastigomonas debruynei, Ancyromonas sigmoides, Apusomonas proboscidea, "Bodo" curvifilus, Bodo saltans, Cafeteria roenbergensis, Caecitellus parvulus, Dimastigella mimosa, Neobodo designis, Neobodo saliens, Parabodo caudatus, Pseudobodo tremulans, Procryptobia sorokini, Rhynchobodo* sp. and *Rhynchomonas nasuta*) was determined, and the ultrastructure of the genetically clearly structured morphospecies *C. parvulus* exemplarily examined, in order to shed light on the nature and relevance of cryptic, respectively pseudo-cryptic (Sáez et al. 2003), species. Speciation can occur both in allopatry (Mayr 1942) and in sympatry (Tautz 2003), most likely along steep ecological gradients (Dieckmann & Doebeli 1999, Doebeli & Dieckmann 2003), wherefore strains were isolated from geographically and ecologically different sample locations in order to find relevant evolutionary patterns.

Three main results have been obtained in this thesis. First, the genetic divergence between the marine and the freshwater lineages of the morphospecies *Ancyromonas sigmoides* strongly suggests that selection acts. Ecological factors, such as salt concentration, can obviously act as major constraints on diversification over evolutionary timescales. Second, the examination of the morphologically indistinguishable and genetically highly diverging strains of the morphospecies *Caecitellus parvulus* revealed several distinguishable traits between the different lineages, leading to the erection of 3—pseudo-cryptic (Sáez et al. 2003)—*Caecitellus* species. All changes in cell morphology of protist morphospecies whose morphology seems to be driven by high selection into adaptive peaks (Nanney 1982), is assumed to be the result of speciation events and should thus reflect disinct species (Andersen 1998, Finlay 2004). Third, the high extent of intraspecific genetic divergence within the morphospecies examined[1], as well as within the GenBANK-dataset, implies a prevalence of cryptic species within protist morphospecies, and in particular within the small heterotrophic flagelattes sequenced in this study ($\leqslant 16$ μm cell size), which appear to be ecological generalists. Estimation of the total protist species number show, that protist species number must be consid-

[1]Summarising basic statistics of the morphospecies examined are given in the Appendix B.

ered to be at least one order of magnitude higher than estimated on morphology alone. Speciation processes might thus be rather coupled to other phenotypic aspects than to morphology.

The findings demonstrate that morphologically indistinguishable morphospecies of protists can harbour very well ecophysiologically and ultrastructural different taxonomic entities—probably species—, and that protist diversity must be considered as grossly underestimated by morphology. High intraspecific genetic divergence should therefore not only be regarded as the result of neutral mutation, according to the neutral theory of molecular evolution (Kimura & Ohta 1971), as suggested by some authors (Fenchel & Finlay 2004). The systematic trend of lumping species under the cloak of morphospecies flies in the face of the evidence for genetic, ecological and biological differences among nominal species. Morphology does not necessarily reflect the expression of a membership to a distinct species. Morphology often seems to represent a fitness of form which is driven by strong selection into adaptive peaks, where it is "frozen and doomed to remain there forever" (Nanney 1982) while other characters can change. Similar morphology might thus be the result of morphological convergent or parallel adaptive evolution (Simpson 1961) or simply of a common ancestor, and thus might mask evolution, as shown for *Ancyromonas* and *Caecitellus*.

DNA barcoding has proved to be useful in circumventing the difficulties posed by the morphological species concept and in distinguishing cryptic species when appearance is deceiving (Blaxter 2003), but DNA barcoding will not resolve the difficult issue of the species concept for protists. Integrative taxonomy will shed light on the extent of intra- and interspecific variation in order to accurately define species boundaries and, hereupon, on all the species hiding behind the facade of a morphospecies (Tautz et al. 2002, Blaxter 2004, Dayrat 2005). What Nanney (1982) called, with a view to ciliates, "the central riddle of heredity"—the relationship between the genotype and the phenotype—is the challenge, protistology has to solve in the near future. This is definitively "no trivial pursuit" (Nanney 2004) and is still unknown for the vast majority of protists but has at least to be determined for protist model organisms, if one will ever get a glance of one of the greatest knowledge gaps in protistology—protist diversity.

Kurzzusammenfassung

Die Diversität von Protisten ist nach wie vor sehr umstritten. Die fundamentale Frage rührt von der Tatsache her, dass es scheinbar weitaus weniger Arten gibt, als nach Hutchinsons und MacArthurs (1959) log-normalem Verhältnis zwischen Artenzahl und Größe a priori zu erwarten wären. Die Morphologie ist seit über 200 Jahren der „Goldstandard" für die Bestimmung von Protisten, weist aber schwerwiegende Mängel auf, da erkennbare morphologische Merkmale oftmals fehlen (Schlegel & Meisterfeld 2003). Der hohe Anteil morphologisch nicht unterscheidbarer – kryptischer – Arten innerhalb nominaler Arten (Sáez & Lozano 2005), führte zu der Vermutung, dass morphologisch bestimmte Arten (Morphoarten – „morphospecies"; Cain 1954) von Protisten lediglich eine Fassade sind, hinter welcher sich eine hohe Anzahl kryptischer Arten verbergen (Cairns 1993).

Da unterschiedliche Arten mit der Zeit genetisch divergieren, wurde die intraspezifische genetische Distanz der kleinen ribosomalen Untereinheit aus Kern-DNA einiger der am weitesten verbreiteten Morphoarten heterotropher Flagellaten (*Amastigomonas debruynei, Ancyromonas sigmoides, Apusomonas proboscidea, "Bodo" curvifilus, Bodo saltans, Cafeteria roenbergensis, Caecitellus parvulus, Dimastigella mimosa, Neobodo designis, Neobodo saliens, Parabodo caudatus, Pseudobodo tremulans, Procryptobia sorokini, Rhynchobodo* sp. und *Rhynchomonas nasuta*), ebenso wie beispielhaft die Ultrastruktur der genetisch deutlich strukturierten Morphoart *C. parvulus* untersucht, um Licht auf die Natur und Relevanz kryptischer, beziehungsweise pseudo-kryptischer (Sáez et al. 2003) Arten zu werfen. Artbildung kann sowohl in Allopatrie (Mayr 1942), als auch in Sympatrie (Tautz 2003) stattfinden und hierbei am leichtesten entlang steiler ökologischer Gradienten (Dieckmann & Doebeli 1999, Doebeli & Dieckmann 2003), weshalb Stämme von geographisch und ökologisch unterschiedlichen Orten isoliert wurden, um eventuell evolutionär relevante Muster zu finden.

Drei wichtige Resultate wurden in dieser Dissertation erzielt. Erstens scheint die genetische Divergenz zwischen Isolaten aus marinen Habitaten und Süßwasser der Morphoart *Ancyromonas sigmoides* das Ergebnis evolutionärer Prozesse zu sein. Ökologische Faktoren wie der Salzgehalt können offensichtlich bedeutend für die Diversifikation in evolutionären Zeiträumen sein. Zweitens ergab die ultrastrukturelle Untersuchung der morphologisch nicht unterscheidbaren und genetisch stark divergierenden Stämme der Morphoart *Caecitellus parvulus* mehrere unterscheidbare Merkmale zwischen den unterschiedlichen Abstammungslinien, welche zu der Beschreibung von 3 – pseudo-kryptischen (Sáez et al. 2003) – *Caecitellus* Arten führte. Alle Änderungen in der Zellmorphologie von Arten, deren Morphologie einem Höchstmaß an Selektionsdruck unterworfen ist, werden als das Resulat von Artbildungsprozessen angesehen (Andersen 1998, Finlay 2004). Drittens impliziert das hohe Maß intraspezifischer, genetischer Divergenz innerhalb der untersuchten Arten[2] sowie des GenBANK-Datensatzes eine große Zahl kryptischer Arten innerhalb von Morphoarten von Protisten, insbesondere

[2]Die zusammengefassten grundlegenden statistischen Werte der untersuchten Morphoarten sind in Anhang B aufgeführt.

innnerhalb der in dieser Dissertation untersuchten kleinen, heterotrophen Flagellaten (Zellgröße $\leqslant 16$ µm), welche als ökologische Generalisten erscheinen. Schätzungen der Gesamtartenzahl zeigten, dass die Anzahl an Protisten eine Größenordnung höher liegen sollte, als auf Grund von Schätzungen, basierend auf Morphoarten, angenommen wird.

Die Ergebnisse zeigen, dass morphologisch nicht unterscheidbare, nominale Arten von Protisten sehr wohl ökophysiologisch und ultrastrukturell unterschiedliche taxonomische Einheiten – vermutlich Arten – beherbergen können, und somit die Diversität von Protisten als deutlich unterschätzt angesehen werden muss. Eine hohe intraspezifische genetische Divergenz sollte demzufolge nicht nur als das Resultat neutraler Mutation, im Sinne der neutralen Theorie molekularer Evolution (Kimura & Ohta 1971), betrachtet werden, wie es einige Autoren vermuten (Fenchel & Finlay 2004). Der systematische Trend, Arten unter dem Deckmantel von Morphoarten zusammenzulegen, widerspricht allen Hinweisen auf unterscheidbare genetische, ökologische und biologische Merkmale innerhalb von Morphoarten. Die Morphologie scheint häufig einer Fitness der Form zu entsprechen, welche durch einen hohen Selektionsdruck in ein Höchstmaß an Anpassung gezwungen wird, um dort bis in alle Ewigkeit zu verharren (Nanney 1982), wohingegen andere Merkmale sich ändern können. Eine ähnliche Morphologie mag demzufolge oftmals eher die Folge von konvergenter oder paralleler, morphologischer Evolution (Simpson 1961) oder einfach nur die Folge eines gemeinsamen Vorfahren sein, als das Resultat der Zugehörigkeit zu einer bestimmten Art. Die Morphologie könnte demnach vielfach evolutionäre Prozesse maskieren, wie *Ancyromonas* und *Caecitellus* zeigten.

„DNA barcoding“ hat sich als nützlich erwiesen, die Schwierigkeiten mit dem morphologischem Artkonzept zu umgehen und kryptische Arten hervorzuheben, wenn die äußere Erscheinung trügt (Blaxter 2003), kann aber alleine genommen das schwierige Problem des Artkonzeptes für Protisten nicht lösen. Integrative Taxonomie ermöglicht es, ein Licht auf das Ausmaß an inter- und intraspezifischer Variabilität werfen, um Artgrenzen exakt bestimmen zu können und in Folge all die Arten aufzudecken, welche sich hinter der Fassade einer Morphoart verbergen (Tautz et al. 2002, Blaxter 2004, Dayrat 2005). Was Nanney (1982) mit Blick auf Ciliaten als das „zentrale Rätsel der Vererbung“ bezeichnete – die Beziehung zwischen Genotyp und Phänotyp –, ist das Problem welches die Protistologie in naher Zukunft lösen muss. Dies ist definitiv nicht trivial (Nanney 2004) und muss für viele Modellorganismen von Protisten noch untersucht werden, ist aber unbedingt nötig, wenn wir jemals einen Ahnung davon bekommnen möchten, was als eine der größten Wissenslücken in der Protistologie bezeichnet werden kann – die Diversität der Protisten.

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Anhang A

Basic statistics of morphospecies with 2 or more sequences. Protist morphospecies with at least 2 available sequences of nuclear small subunit ribosomal DNA (SSU rDNA $\geqslant 1.5$ kb) have been retrieved from GenBANK excluding groups containing multicellular organisms (Fungi, Metazoa, Rhodophyta and Viridiplantae)—in total 386 nominal species with 1514 sequences. Number of sequences (n), minimal (Min.) and maximal (Max.) p-distance, mean p-distance (Mean), standard deviation (S.D.), skewness (Skew), kurtosis (Kurt), 25 % quartile (Q_1), median p-distance (Median) and 75 % quartile (Q_3).

Species	n	Min.†	Max.†	Mean†	S.D.†	Skew	Kurt	Q_1†	Median†	Q_3†
Acanthamoeba astronyxis	2			0.00						
Acanthamoeba castellanii	12	0.04	6.07	2.51	1.64	0.04	0.60	1.69	2.19	2.42
Acanthamoeba culbertsoni	2			0.00						
Acanthamoeba divionensis	2			0.00						
Acanthamoeba griffini	2			0.00						
Acanthamoeba hatchetti	6	0.22	7.67	4.34	2.45	-0.02	-0.99	1.97	4.06	6.84
Acanthamoeba lenticulata	12	0.00	27.95	5.94	9.10	0.04	0.90	0.34	0.62	11.22
Acanthamoeba lugdunensis	2			0.00						
Acanthamoeba palestinensis	4	0.26	8.61	5.34	3.10	-0.27	-0.03	2.20	4.17	7.44
Acanthamoeba polyphaga	11	0.04	8.05	3.92	2.76	0.01	-1.54	1.29	2.58	7.22
Acanthamoeba rhysodes	3	1.43	2.73	2.01	0.66	0.58		0.36	1.66	2.52
Acanthamoeba triangularis	2			0.00						
Adelina bambarooniae	2			0.00						
Akashiwo sanguinea	3	0.00	1.14	0.75	0.65	-1.15		0.00	0.56	1.13
Alexandrium affine	2			0.00						
Alexandrium catenella	17	0.00	1.03	0.17	0.25	0.02	0.44	0.00	0.06	0.17
Alexandrium fraterculus	3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Alexandrium minutum	3	0.17	0.40	0.26	0.12	0.87		0.04	0.20	0.35
Alexandrium ostenfeldii	5	0.06	0.62	0.48	0.17	-0.36	3.34	0.37	0.56	0.58
Alexandrium tamarense	37	0.00	4.34	1.55	1.15	0.00	-0.32	0.46	1.83	2.11
Alexandrium tamiyavanichi	10	0.00	0.33	0.12	0.09	0.04	-0.13	0.06	0.11	0.17
Alexandrium taylori	3	0.00	0.06	0.04	0.03	-1.15		0.00	0.03	0.06
Amastigomonas debruynei	3	0.06	0.23	0.15	0.09	-1.15		0.12	0.17	0.20
Ammonia beccarii	2			0.00						
Amphidinium carterae	3	0.00	0.06	0.04	0.03	-1.15		0.00	0.03	0.06
Amphisorus hemprichii	2			0.00						
Ancyromonas sigmoides	9	0.00	18.73	10.81	7.57	-0.03	-1.64	1.98	15.30	17.42
Apusomonas proboscidea	2			1.52						
Astasia curvata	4	0.16	8.56	5.72	4.20	-0.32	-1.86	0.30	8.25	8.46
Asterionellopsis glacialis	2			0.00						
Aulacoseira ambigua	6	0.00	0.06	0.03	0.03	-0.02	-2.30	0.00	0.03	0.06
Aulacoseira baicalensis	3	0.06	0.67	0.43	0.33	-1.01		0.01	0.31	0.64
Aulacoseira granulata	3	0.50	1.18	0.87	0.34	-0.54		0.12	0.71	1.11
Aulacoseira islandica	3	0.00	2.12	0.71	1.23	1.15		0.00	0.00	1.59
Aulacoseira nyassensis	2			0.00						
Aulacoseira skvortzowii	2			0.00						
Aulacoseira subarctica	4	0.06	0.95	0.49	0.34	0.03	-1.61	0.15	0.31	0.70
Aureococcus anophagefferens	7	0.00	0.39	0.11	0.18	0.10	-1.06	0.00	0.00	0.10
Babesia bigemina	3	0.00	0.47	0.31	0.27	-1.15		0.00	0.23	0.47
Babesia bovis	4	0.73	3.29	2.05	1.27	-0.02	-3.05	0.74	1.25	3.14
Babesia caballi	3	0.42	2.55	1.82	1.21	-1.15		0.10	1.45	2.53
Babesia crassa	2			0.00						
Babesia divergens	7	0.00	2.21	0.89	0.59	0.05	-0.49	0.36	0.80	1.23
Babesia equi	4	0.00	0.40	0.24	0.13	-0.39	2.66	0.11	0.23	0.29
Babesia felis	12	0.00	0.30	0.15	0.06	0.00	-0.46	0.12	0.13	0.19
Babesia gibsoni	6	0.00	10.27	5.29	4.38	-0.02	-2.11	0.06	5.35	8.88
Babesia leo	2			0.00						
Babesia microti	11	0.00	2.53	0.90	0.76	0.03	-0.39	0.12	0.74	1.19
Babesia motasi	3	0.00	0.12	0.08	0.07	-1.15		0.00	0.06	0.12

† Values in %.

Species	*n*	Min.†	Max.†	Mean†	S.D.†	Skew	Kurt	Q_1†	Median†	Q_3†
Babesia odocoilei	6	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Babesia ovis	3	0.30	0.54	0.40	0.13	0.88		0.07	0.33	0.49
Balamuthia mandrillaris	5	0.00	0.36	0.14	0.18	0.10	-2.28	0.00	0.00	0.36
Blastocystis hominis	31	0.11	15.94	9.87	4.45	-0.00	-0.06	8.37	11.61	12.75
Bodanella lauterborni	2			0.00						
Bodo curvifilus	2			14.30						
Bodo saltans	23	0.00	9.62	4.00	1.90	0.01	1.69	3.16	3.72	4.07
Bolidomonas pacifica	6	0.11	2.86	1.61	1.24	-0.06	-2.06	0.17	2.42	2.53
Caecitellus parvulus	11	0.00	5.60	2.91	2.48	-0.01	-1.91	0.03	4.44	5.20
Cafeteria roenbergensis	5	0.00	0.68	0.28	0.27	0.17	-1.27	0.08	0.12	0.54
Caryospora bigenetica	2			0.00						
Cercomonas longicauda	3	1.01	1.37	1.21	0.18	-0.58		0.25	1.13	1.34
Chaetoceros calcitrans	2			0.00						
Chaetoceros gracilis	2			0.00						
Chaetoceros muelleri	2			0.00						
Chilodonella uncinata	4	0.24	0.49	0.37	0.10	0.01	-1.98	0.27	0.30	0.43
Chlamydaster sterni	2			0.00						
Chlamydodon excocellatus	4	0.00	0.12	0.06	0.04	-0.00	2.49	0.03	0.06	0.06
Chlorarachnion reptans	4	0.79	27.82	18.74	13.69	-0.32	-1.87	1.07	27.38	27.55
Cochlodinium polykrikoides	5	0.00	0.06	0.02	0.03	0.10	-2.28	0.00	0.00	0.06
Colacium mucronatum	2			0.00						
Costaria costata	3	0.06	0.44	0.28	0.20	-0.84		0.01	0.20	0.42
Crithidia oncopelti	2			0.00						
Cryothecomonas aestivalis	2			0.00						
Cryptomonas ovata	2			0.00						
Cryptomonas pyrenoidifera	2			0.00						
Cryptosporidium baileyi	3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Cryptosporidium felis	2			0.00						
Cryptosporidium muris	8	0.00	1.57	0.69	0.43	0.02	-0.43	0.36	0.55	0.96
Cryptosporidium parvum	43	0.00	21.10	1.43	2.30	0.01	17.99	0.23	0.40	1.95
Cryptosporidium serpentis	5	0.00	0.34	0.16	0.15	0.06	-1.66	0.00	0.11	0.29
Cryptosporidium wrairi	2			0.00						
Cyclospora cercopitheci	2			0.00						
Cyclotella meneghiniana	7	0.06	0.62	0.33	0.18	-0.03	-0.88	0.20	0.34	0.41
Cyclotella scaldensis	2			0.00						
Cylindrotheca closterium	2			0.00						
Cytauxzoon felis	2			0.00						
Cytauxzoon manul	2			0.00						
Dasytricha ruminantium	2			0.00						
Diacronema vlkianum	2			0.00						
Dictyopteris divaricata	2			0.00						
Dictyopteris polypodioides	2			0.00						
Dictyopteris prolifera	3	0.00	1.82	1.21	1.05	-1.15		0.00	0.91	1.82
Dictyostelium discoideum	2			0.00						
Dictyota dichotoma	4	0.00	0.45	0.22	0.25	0.00	-3.33	0.00	0.00	0.45
Dientamoeba fragilis	2			0.00						
Dilophus okamurae	4	0.00	0.28	0.14	0.12	0.00	-2.53	0.03	0.06	0.23
Dimastigella mimosa	2			0.05						
Dimastigella trypaniformis	3	1.08	1.70	1.42	0.32	-0.49		0.27	1.27	1.64
Dinenympha exilis	2			0.00						
Dinophysis acuminata	2			0.00						
Dinophysis norvegica	3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Diplonema ambulator	2			0.00						
Distigma curvatum	4	31.78	40.96	36.70	3.66	-0.11	-1.68	32.46	36.31	39.01
Distigma gracilis	2			0.00						
Distigma proteus	3	0.41	0.41	0.41	0.00	-1.15		0.10	0.41	0.41
Distromium decumbens	3	0.17	5.48	3.71	3.06	-1.15		0.04	2.82	5.48
Ditylum brightwellii	4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Echinamoeba thermarum	8	0.55	3.41	2.00	0.97	0.00	-1.39	0.88	1.94	2.89
Ectocarpus siliculosus	2			0.00						
Eimeria mitis	2			0.00						
Eimeria tenella	3	0.00	0.29	0.19	0.17	-1.15		0.00	0.14	0.29
Emiliania huxleyi	3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Entamoeba coli	2			0.00						

† Values in %.

Species	*n*	Min.†	Max.†	Mean†	S.D.†	Skew	Kurt	Q_1†	Median†	Q_3†
Entamoeba histolytica	3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Entamoeba invadens	2			0.00						
Entomoneis alata	2			0.00						
Entosiphon sulcatum	2			0.00						
Euglena acus	6	0.00	9.43	4.18	3.73	-0.00	-1.81	0.03	5.04	8.12
Euglena agilis	2			0.00						
Euglena anabaena	4	0.00	12.04	6.02	6.59	0.00	-3.33	0.00	0.00	12.03
Euglena deses	4	7.77	11.57	10.36	1.37	-0.58	3.36	9.00	10.37	11.12
Euglena geniculata	6	0.00	23.61	12.63	8.29	0.02	-1.19	7.81	9.53	22.72
Euglena gracilis	6	0.00	9.95	5.47	4.67	-0.04	-2.12	0.13	7.03	9.64
Euglena laciniata	2			0.00						
Euglena longa	5	0.00	3.41	1.36	1.67	0.10	-2.27	0.00	0.13	3.26
Euglena mutabilis	8	0.04	16.66	7.50	5.27	0.03	-1.08	2.58	7.09	9.42
Euglena myxocylindracea	2			0.00						
Euglena oxyuris	2			0.00						
Euglena spirogyra	4	0.05	2.51	1.84	0.91	-0.69	4.65	0.99	1.99	2.28
Euglena stellata	6	0.14	25.99	17.82	7.62	-0.12	0.30	10.40	19.51	24.58
Euglena tripteris	3	0.00	2.56	1.70	1.47	-1.15		0.00	1.27	2.55
Euglena tristella	2			0.00						
Euglena viridis	8	0.05	24.56	17.35	6.95	-0.12	1.86	16.80	19.91	21.25
Euglypha filifera	2			0.00						
Euglypha rotunda	4	0.50	2.02	1.55	0.60	-0.45	0.95	0.84	1.79	1.90
Eunotia pectinalis	2			0.00						
Euplotes aediculatus	3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Euplotes charon	2			0.00						
Euplotes eurystomus	2			0.00						
Euplotes harpa	3	0.05	0.11	0.07	0.03	1.15		0.01	0.05	0.09
Euplotes magnicirratus	3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Euplotes minuta	4	0.00	0.05	0.03	0.03	0.00	-3.33	0.00	0.00	0.05
Euplotes rariseta	2			0.00						
Euplotes vannus	4	0.00	1.14	0.57	0.63	0.00	-3.33	0.00	0.00	1.14
Euplotes woodruffi	2			0.00						
Eutintinnus pectinis	4	0.00	3.51	1.76	1.72	-0.00	-3.24	0.15	0.29	3.24
Eutreptia viridis	2			0.00						
Exanthemachrysis gayraliae	5	0.00	4.53	1.86	1.97	0.10	-2.17	0.23	0.57	4.00
Favella ehrenbergii	5	0.06	0.24	0.15	0.07	-0.06	-0.92	0.09	0.18	0.18
Fragilaria striatula	2			0.00						
Fucus distichus	2			0.00						
Geleia simplex	6	0.00	0.07	0.02	0.03	0.11	-1.62	0.00	0.00	0.07
Geminigera cryophila	4	0.00	0.17	0.09	0.10	0.00	-3.33	0.00	0.00	0.17
Gephyrocapsa oceanica	3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Gonyaulax polyedra	2			0.00						
Gonyaulax spinifera	2			0.00						
Grammatophora oceanica	2			0.00						
Gromia oviformis	5	4.58	8.74	7.03	1.49	-0.11	-1.02	5.46	7.19	8.15
Guinardia delicatula	2			0.00						
Gymnodinium beii	5	0.00	0.61	0.24	0.32	0.10	-2.28	0.00	0.00	0.61
Gymnodinium catenatum	4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Gymnodinium mikimotoi	3	0.06	0.17	0.11	0.06	-0.00		0.01	0.08	0.15
Gymnodinium sanguineum	3	0.06	0.85	0.55	0.43	-1.07		0.01	0.40	0.82
Gymnophrys cometa	2			0.00						
Gyrodinium aureolum	2			0.00						
Gyrodinium instriatum	3	0.18	0.40	0.27	0.12	0.84		0.04	0.20	0.36
Halteria grandinella	5	0.12	0.46	0.26	0.12	0.11	-1.32	0.18	0.18	0.35
Haplosporidium costale	2			0.00						
Haplosporidium nelsoni	3	0.17	0.69	0.50	0.28	-1.10		0.04	0.40	0.68
Hartmannella vermiformis	2			0.00						
Heliophrya erhardi	5	0.18	0.37	0.27	0.08	0.06	-1.67	0.18	0.24	0.34
Heribaudiella fluviatilis	5	0.00	0.73	0.30	0.36	0.09	-2.26	0.00	0.06	0.71
Herpetomonas roitmani	2			0.00						
Heteramoeba clara	2			0.00						
Heterocapsa triquetra	4	0.00	0.17	0.09	0.09	0.00	-3.33	0.00	0.00	0.17
Heteromita globosa	2			0.00						
Heterosigma akashiwo	5	0.00	0.40	0.13	0.15	0.30	0.87	0.03	0.06	0.11

† Values in %.

Species	*n*	Min.†	Max.†	Mean†	S.D.†	Skew	Kurt	Q_1†	Median†	Q_3†
Hyphochytrium catenoides	2			0.00						
Ichthyobodo necator	2			0.00						
Isospora belli	2			0.00						
Jakoba libera	2			0.00						
Karenia brevis	3	0.06	0.29	0.19	0.12	-0.86		0.01	0.14	0.27
Karlodinium micrum	7	0.00	0.17	0.09	0.06	-0.06	-1.01	0.00	0.11	0.11
Laboea strobila	4	0.00	0.12	0.07	0.04	-0.10	-0.12	0.03	0.06	0.09
Lagenidium giganteum	2			0.00						
Laminaria japonica	2			0.00						
Lecudina polymorpha	2			0.00						
Leishmania tarentolae	2			0.00						
Lepocinclis ovum	2			0.00						
Leptocylindrus danicus	2			0.00						
Leptolegnia chapmanii	2			0.00						
Lingulodinium polyedrum	2			0.00						
Lobophora variegata	6	0.00	0.78	0.39	0.25	-0.06	-0.79	0.07	0.44	0.61
Mallomonas striata	2			0.00						
Massisteria marina	7	0.00	1.50	0.64	0.52	0.04	-1.17	0.18	0.47	0.84
Melosira varians	3	0.06	0.57	0.40	0.29	-1.15		0.02	0.32	0.57
Menoidium gibbum	3	0.17	0.49	0.34	0.16	-0.23		0.04	0.26	0.45
Metacylis angulata	4	0.00	0.91	0.44	0.42	0.03	-2.97	0.03	0.12	0.77
Metopus palaeformis	4	0.12	0.38	0.25	0.12	0.02	-1.88	0.12	0.24	0.32
Minchinia teredinis	2			0.00						
Moneuplotes crassus	8	0.00	0.93	0.42	0.23	0.03	0.31	0.28	0.44	0.49
Monocercomonas ruminantium	2			0.00						
Monosiga brevicollis	3	0.06	0.17	0.11	0.06	-0.02		0.01	0.09	0.16
Naegleria australiensis	3	0.10	0.30	0.20	0.10	-0.01		0.03	0.15	0.28
Naegleria clarki	5	0.06	0.33	0.22	0.08	-0.02	0.10	0.18	0.18	0.26
Naegleria fowleri	2			0.00						
Nannochloropsis gaditana	10	0.00	0.11	0.02	0.04	0.09	2.17	0.00	0.00	0.00
Nannochloropsis granulata	7	0.00	0.06	0.03	0.03	0.01	-2.21	0.00	0.00	0.06
Nannochloropsis oceanica	6	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Nannochloropsis oculata	3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Nannochloropsis salina	9	0.00	0.11	0.02	0.05	0.08	-0.06	0.00	0.00	0.00
Nanofrustulum shiloi	2			0.00						
Navicula pelliculosa	2			0.00						
Neobodo designis	20	0.25	29.17	10.85	4.88	0.00	0.07	6.95	11.89	13.73
Neobodo saliens	2			11.81						
Neoparamoeba aestuarina	4	1.01	3.27	2.29	0.96	-0.09	-2.30	1.27	1.83	3.06
Neoparamoeba pemaquidensis	9	0.00	2.67	1.60	0.65	-0.02	-0.11	1.14	1.66	1.99
Neospora caninum	5	0.00	0.16	0.05	0.07	0.13	-1.64	0.00	0.00	0.11
Nuclearia moebiusi	2			0.00						
Nuclearia simplex	2			0.00						
Nyctotherus ovalis	5	0.06	3.44	1.54	1.56	0.09	-2.24	0.34	0.43	3.26
Oxyrrhis marina	2			0.00						
Pachydictyon coriaceum	4	0.00	0.34	0.17	0.12	0.01	-0.17	0.06	0.11	0.22
Padina arborescens	2			0.00						
Padina australis	3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Padina crassa	3	0.06	2.43	1.62	1.36	-1.15		0.01	1.21	2.42
Padina japonica	3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Parabodo caudatus	7	1.98	5.28	2.96	1.42	0.01	-0.73	2.09	2.55	4.07
Parabodo nitrophilus	2			0.00						
Paraflabellula hoguae	2			0.00						
Paramecium calkinsi	2			0.00						
Paramecium nephridiatum	2			0.00						
Paramecium tetraurelia	2			0.00						
Paraphysomonas foraminifera	2			0.00						
Paraphysomonas imperforata	2			0.00						
Paraphysomonas vestita	2			0.00						
Pavlova gyrans	5	0.00	0.28	0.15	0.10	-0.04	-1.12	0.03	0.17	0.20
Pavlova lutheri	4	0.00	6.68	3.23	3.54	0.00	-3.32	0.00	0.00	6.35
Pavlova pinguis	7	0.00	3.19	1.38	1.12	0.07	-1.11	0.46	0.96	1.70
Pavlova salina	3	0.29	2.91	1.97	1.46	-1.13		0.07	1.49	2.86
Pentatrichomonas hominis	2			0.00						

† Values in %.

Species	*n*	Min.†	Max.†	Mean†	S.D.†	Skew	Kurt	Q_1†	Median†	Q_3†
Percolomonas cosmopolitus	3	0.08	40.75	25.64	22.26	-1.10		18.09	36.10	38.43
Peridinium willei	2			0.00						
Perkinsus andrewsi	2			0.00						
Perkinsus marinus	4	0.19	0.83	0.50	0.22	0.04	0.18	0.26	0.50	0.57
Perkinsus mediterraneus	6	0.00	0.28	0.14	0.09	-0.05	-0.56	0.03	0.14	0.21
Petalomonas cantuscygni	2			0.00						
Pfiesteria piscicida	27	0.00	0.34	0.03	0.06	0.01	2.36	0.00	0.00	0.05
Pfiesteria shumwayae	2			0.00						
Phacus acuminatus	3	15.40	19.90	17.79	2.26	-0.36		3.85	16.73	19.44
Phacus alatus	2			0.00						
Phacus brachykentron	2			0.00						
Phacus caudatus	2			0.00						
Phacus orbicularis	3	0.00	4.98	3.32	2.87	-1.15		0.00	2.49	4.98
Phacus oscillans	3	0.00	0.70	0.47	0.40	-1.15	0.00	0.35	0.70	
Phacus pleuronectes	2			0.00						
Phacus pseudonordstedtii	3	0.00	7.71	5.14	4.45	-1.15		0.00	3.86	7.71
Phacus pusillus	4	0.14	2.49	1.31	1.20	-0.00	-3.29	0.16	0.32	2.37
Phacus similis	4	0.00	0.19	0.09	0.10	0.00	-3.33	0.00	0.00	0.19
Phacus striatus	2			0.00						
Phacus triqueter	2			0.00						
Phaeocystis antarctica	5	0.00	0.28	0.13	0.11	0.07	-1.76	0.06	0.06	0.22
Phaeocystis globosa	9	0.00	1.31	0.40	0.40	0.03	-1.06	0.06	0.22	0.67
Phaeocystis pouchetii	3	0.00	0.39	0.26	0.22	-1.15		0.00	0.19	0.39
Phaeodactylum tricornutum	2			0.00						
Phaeomonas parva	2			0.00						
Phytomonas serpens	3	0.05	0.19	0.11	0.07	0.62		0.01	0.07	0.17
Phytophthora capsici	2			0.00						
Phytophthora infestans	2			0.00						
Phytophthora megasperma	2			0.00						
Phytophthora nicotianae	2			0.00						
Phytophthora palmivora	2			0.00						
Phytophthora tropicalis	2			0.00						
Pirsonia formosa	3	0.06	0.11	0.08	0.03	1.15		0.01	0.06	0.10
Plasmodium berghei	2			0.00						
Plasmodium cynomolgi	2			0.00						
Plasmodium falciparum	2			0.00						
Plasmodium juxtanucleare	2			0.00						
Plasmodium knowlesi	12	0.00	10.26	1.94	3.38	0.05	1.32	0.18	0.44	1.12
Plasmodium malariae	2			0.00						
Plasmodium ovale	7	0.19	3.11	1.81	1.24	-0.03	-2.02	0.48	2.63	2.83
Plasmodium vivax	10	0.09	17.30	8.16	5.75	-0.01	-1.24	0.56	9.17	12.18
Pleurochrysis carterae	2			0.00						
Pleurosira laevis	2			0.00						
Polarella glacialis	2			0.00						
Polyplastron multivesiculatum	2			0.00						
Polypodochrysis teissieri	2			0.00						
Proboscia alata	2			0.00						
Procryptobia sorokini	4	0.10	0.75	0.45	0.25	-0.14	-1.19	0.27	0.52	0.59
Proleptomonas faecicola	2			0.00						
Prorocentrum minimum	3	0.00	0.23	0.15	0.13	-1.15		0.00	0.11	0.23
Protoceratium reticulatum	2			0.00						
Prymnesium patelliferum	2			0.00						
Psalteriomonas lanterna	2			0.00						
Pseudobodo tremulans	2			0.23						
Pseudotrypanosoma giganteum	5	0.26	0.59	0.43	0.12	0.03	-1.42	0.33	0.39	0.52
Pyrenomonas salina	2			0.00						
Pyrsonympha grandis	4	0.81	8.67	7.07	3.10	-0.78	5.56	4.14	8.46	8.51
Pythium insidiosum	3	0.05	0.11	0.07	0.03	1.15		0.01	0.05	0.10
Pythium splendens	2			0.00						
Pythium vexans	2			0.00						
Raphidiophrys ambigua	2			0.00						
Reclinomonas americana	2			0.00						
Rhabdomonas costata	2			0.00						
Rhabdomonas incurva	2			0.00						

† Values in %.

Species	*n*	Min.†	Max.†	Mean†	S.D.†	Skew	Kurt	Q_1†	Median†	Q_3†
Rhizosolenia setigera	4	0.00	12.50	9.13	4.65	-0.68	4.52	4.68	10.14	11.39
Rhodomonas mariana	2			0.00						
Rhynchomonas nasuta	16	0.00	6.49	3.59	1.88	-0.01	-1.03	1.74	4.20	5.04
Saprolegnia parasitica	3	0.23	0.79	0.60	0.33	-1.15		0.06	0.51	0.79
Sarcocystis fusiformis	3	0.06	0.32	0.21	0.13	-0.87		0.02	0.16	0.30
Sarcocystis hirsuta	5	0.06	1.00	0.57	0.34	-0.03	-1.45	0.23	0.52	0.87
Sarcocystis sinensis	2			0.00						
Sarcocystis singaporensis	4	0.55	5.27	2.86	2.34	0.00	-3.24	0.61	0.98	4.85
Scrippsiella trochoidea	3	0.00	0.17	0.10	0.09	-0.63		0.00	0.06	0.16
Scytosiphon lomentaria	2			0.00						
Sellaphora laevissima	2			0.00						
Sellaphora pupula	11	0.00	1.93	0.94	0.58	-0.02	-1.01	0.13	1.17	1.29
Skeletonema costatum	4	0.00	0.23	0.17	0.09	-0.68	4.42	0.09	0.18	0.22
Skeletonema menzelii	2			0.00						
Skeletonema pseudocostatum	3	0.00	1.45	0.97	0.84	-1.15		0.00	0.73	1.45
Skeletonema subsalsum	2			0.00						
Snyderella tabogae	5	0.07	0.60	0.37	0.17	-0.07	-0.89	0.23	0.33	0.49
Sorites orbiculus	2			0.00						
Sorogena stoianovitchae	4	0.23	0.29	0.26	0.03	-0.00	-3.33	0.23	0.23	0.29
Spatoglossum crassum	2			0.00						
Spatoglossum pacificum	4	0.00	0.06	0.03	0.03	0.00	-3.33	0.00	0.00	0.06
Spongomonas minima	2			0.00						
Spongospora subterranea	4	0.27	8.06	5.27	3.51	-0.31	-1.65	0.79	6.99	7.50
Streblomastix strix	2			0.00						
Strombomonas acuminata	2			0.00						
Stylonychia mytilus	2			0.00						
Symbiomonas scintillans	2			0.00						
Tetrahymena australis	2			0.00						
Tetrahymena canadensis	2			0.00						
Tetrahymena capricornis	2			0.00						
Tetrahymena hegewishii	2			0.00						
Tetrahymena hyperangularis	2			0.00						
Tetrahymena nanneyi	2			0.00						
Tetrahymena patula	2			0.00						
Tetrahymena pyriformis	2			0.00						
Tetrahymena tropicalis	2			0.00						
Tetramitus thermacidophilus	2			0.00						
Thalassicolla nucleata	5	0.45	1.81	1.31	0.53	-0.18	-1.14	0.62	1.53	1.67
Thalassiosira pseudonana	3	0.28	4.91	3.30	2.62	-1.15		0.07	2.50	4.86
Thalassiosira punctigera	2			0.00						
Thalassiosira rotula	4	0.17	0.56	0.36	0.14	0.00	0.03	0.22	0.33	0.42
Thalassiosira weissflogii	3	0.11	0.45	0.32	0.18	-1.00		0.03	0.25	0.44
Thaumatomonas seravini	2			0.00						
Theileria buffeli	4	0.00	0.40	0.26	0.18	-0.31	-1.62	0.03	0.34	0.37
Theileria ovis	3	0.06	0.17	0.11	0.06	0.05		0.01	0.09	0.16
Theileria parva	3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Theileria sergenti	3	0.34	1.49	1.04	0.61	-0.97		0.09	0.81	1.44
Tintinnopsis tubulosoides	4	0.00	0.23	0.12	0.07	-0.00	2.50	0.06	0.12	0.12
Tokophrya lemnarum	2			0.00						
Toxoplasma gondii	8	0.00	1.02	0.37	0.33	0.05	-0.88	0.12	0.22	0.62
Trachelomonas echinata	2			0.00						
Trachelomonas hispida	2			0.00						
Trachelomonas pertyi	2			0.00						
Trachelomonas volvocina	2			0.00						
Trepomonas agilis	2			0.00						
Trichomitus batrachorum	2			0.00						
Trichomonas vaginalis	5	0.13	1.20	0.61	0.35	0.07	-1.18	0.33	0.40	0.90
Trichonympha magna	7	0.20	1.05	0.58	0.24	0.03	-0.70	0.39	0.56	0.74
Trimastix pyriformis	2			0.00						
Tritrichomonas foetus	6	0.00	0.85	0.22	0.29	0.14	-0.20	0.00	0.05	0.52
Trypanosoma avium	5	0.00	0.51	0.28	0.17	-0.01	-0.63	0.14	0.24	0.38
Trypanosoma binneyi	2			0.00						
Trypanosoma brucei	3	0.05	0.09	0.06	0.03	1.15		0.01	0.05	0.08
Trypanosoma congolense	8	0.14	21.31	6.16	6.10	0.09	0.68	1.58	4.24	7.77

† Values in %.

Species	*n*	Min.†	Max.†	Mean†	S.D.†	Skew	Kurt	Q_1†	Median†	Q_3†
Trypanosoma cruzi	57	0.00	2.47	1.13	0.52	-0.00	-0.67	0.77	1.12	1.53
Trypanosoma dionisii	2			0.00						
Trypanosoma equiperdum	2			0.00						
Trypanosoma evansi	3	0.00	0.18	0.08	0.10	0.90		0.00	0.02	0.15
Trypanosoma grayi	2			0.00						
Trypanosoma grosi	3	1.21	1.28	1.24	0.03	0.66	0.30	1.22	1.26	
Trypanosoma lewisi	2			0.00						
Trypanosoma mega	3	0.00	1.99	1.32	1.14	-1.15		0.00	0.99	1.99
Trypanosoma otospermophili	2			0.00						
Trypanosoma rangeli	8	0.00	0.77	0.34	0.22	-0.00	-0.49	0.23	0.27	0.50
Trypanosoma rotatorium	2			0.00						
Trypanosoma simiae	4	0.09	3.11	1.87	1.30	-0.10	-2.30	0.56	1.07	2.96
Trypanosoma theileri	3	0.00	0.68	0.45	0.39	-1.15		0.00	0.34	0.68
Trypanosoma varani	2			0.00						
Ulkenia profunda	2			0.00						
Uronema marinum	2			0.00						
Zonaria diesingiana	5	0.00	1.52	0.62	0.74	0.10	-2.27	0.06	0.06	1.47

† Values in %.

Anhang B

Basic statistics of all morphospecies studied (*Amastigomonas debruynei, Ancyromonas sigmoides, Apusomonas proboscidea, "Bodo" curvifilus, Bodo saltans, Cafeteria roenbergensis, Caecitellus parvulus, Dimastigella mimosa, Neobodo designis, Neobodo saliens, Parabodo caudatus, Pseudobodo tremulans, Procryptobia sorokini, Rhynchobodo* sp., *Rhynchomonas nasuta*, as well as *Percolomonas cosmopolitus* (Scheckenbach 2003)): mean *p*-distance = 5.98 %, median *p*-distance = 8.24 %, Q_1 = 4.42 %, Q_3 = 36.26 %, mode = 0.00 %, skewness = 1.59, kurtosis = 3.75. No morphospecies is genetically identical (0.00 % *p*-distance) and only 13.34 % contain identical sequences, whereas 86.67 % contain cryptic species (OTU $\leqslant$ 0.50 % *p*-distance) and 40 % are para- or polyphyletic. 15 morphospecies (*S*) plus one strain of *Rhynchobodo* sp. have been sequenced with 63 complete 18S rDNA, 37 D3–D5 28S rDNA and 26 ITS1 DNA sequences. The observed (S_{obs}) number of cryptic species lies between 79 (OTU $\leqslant$ 0.50 % *p*-distance; $S_{obs}^{0.50\,\%}$) and 100 (OTU = 0.00 % *p*-distance; $S_{obs}^{0.00\,\%}$). The estimated number (Chao1) of cryptic species lies between 199 (OTU $\leqslant$ 0.50 % *p*-distance; $S_{Chao1}^{0.50\,\%}$) and 606 (OTU = 0.00 % *p*-distance; $S_{Chao1}^{0.00\,\%}$). The total number of species should therefore be 15.9–40.4 $\times$ ($S_{obs}^{0.50\,\%}/S$–$S_{Chao1}^{0.00\,\%}/S$) higher than the number of morphospecies.

Köln, 27. Oktober 2005

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 Abbildungen und Tabellen –, 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 den auf der folgenden Seite angegebenen Teilpublikationen – noch nicht veröffentlicht worden ist sowie, dass ich eine solche Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde.

Die Bestimmungen dieser Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Prof. Dr. Hartmut Arndt betreut worden.

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Teilpublikationen:

- 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

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- Scheckenbach F, Arndt H. (in preparation). Where are all the protists?

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