UNIVERSITY OF COLOGNE

Biodiversity and Evolution of Protists in the Atacama Desert

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„In den kleinsten Dingen zeigt die Natur
die allergrößten Wunder“

—Carl von Linné

UNIVERSITY OF COLOGNE

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Abstract

The Atacama Desert, an isolated habitat in the northern part of Chile, contains unique geological and hydrological conditions. The location of hypersaline inland waters (salars) and aquatic systems like rivers within this extreme habitat makes it particularly interesting to study aquatic microbial diversity and adaptation. The main objective was to enlarge the knowledge about protists' diversity, adaptation, community composition and distribution in extreme environments of the Atacama Desert. A wide range of different habitats were sampled. The comparison of metabarcoding studies together with studies of isolated protist cultures from the different extreme habitats were used to analyse the biodiversity and distribution of protists. Especially, representatives of the recently discovered class Placididea within the supergroup of Stramenopiles were isolated from numerous different salars and they turned out to be highly adapted to these environments. Further, novel organisms of Percolomonadida and Cafeteriaceae were identified, isolated, and described from these extreme environments in the frame of the present studies. Placididea were additionally used as a model group for hypersaline environments to investigate global distribution patterns of protists and to receive the first results on the evolution and divergence of heterotrophic protists in combination with the geological evolution. Investigations of genotypes showed a unique community composition in nearly every salar and even among different microhabitats within one salar. The relatively low dispersal rate and the ability of many protist species for a relatively fast genetic adaptation at high generation times suggest that the salars should be considered as hot spots of protist evolution, especially for representatives of Stramenopiles and Alveolata.

UNIVERSITÄT ZU KÖLN

Mathematisch-Naturwissenschaftliche Fakultät

Kurzzusammenfassung

Die Atacama-Wüste, ein isolierter Lebensraum im Norden Chiles, bietet einzigartige geologische und hydrologische Bedingungen. Die Lage hypersaliner Binnengewässer (Salare) und aquatischer Systeme wie Flüsse innerhalb dieses extremen Habitats macht es besonders interessant, die aquatische mikrobielle Diversität und Anpassung zu untersuchen. Das Hauptziel bestand darin, das Wissen über die Diversität, Anpassung, Zusammensetzung und Verteilung der Gemeinschaft in extremen Umgebungen von Protisten zu erweitern, veranschaulicht am Beispiel der Atacama-Wüste. Verschiedene Umgebungen in der Atacama-Wüste wurden beprobt, um ein breites Spektrum unterschiedlicher Lebensräume abzudecken. Der Vergleich von Metabarcoding-Studien zusammen mit Studien isolierter Protistenkulturen aus den verschiedenen Extremhabitaten gab Aufschluss über die Biodiversität und Verbreitung der Protisten. Vertreter der erst kürzlich entdeckten Klasse Placididea innerhalb der Supergruppe der Stramenopilen wurden aus zahlreichen unterschiedlichen Salaren isoliert und erwiesen sich als hochgradig an diese Umgebungen angepasst. Im Zusammenhang mit dieser Arbeit wurden aus diesen extremen Umgebungen weitere neuartige Organismen von Percolomonadida und Cafeteriaceae identifiziert, isoliert und beschrieben. Placididea wurden zusätzlich als Modellgruppe für hypersaline Umgebungen verwendet, um globale Verteilungsmuster von Protisten zu untersuchen und erste Ergebnisse zur Evolution und Divergenz heterotropher Protisten in Kombination mit geologischen Veränderungen zu erhalten. Genotypenuntersuchungen zeigten eine einzigartige Gemeinschaftszusammensetzung in fast jedem Salar und sogar zwischen verschiedenen Mikrohabitaten innerhalb eines Salars. Die relativ geringe

Verbreitungsrate und die Fähigkeit vieler Protistenarten zu einer relativ schnellen genetischen Anpassung bei hohen Generationszeiten legen nahe, dass die Salare als „Hot Spots“ der Protistenevolution betrachtet werden sollten, insbesondere für Vertreter der Stramenopiles und Alveolata.

Contents

General Introduction

The Atacama Desert – an extreme environment

The Atacama Desert, one of the driest places worldwide, offers a unique setting for the investigation of aquatic environments (Berger and Cooke, 1997; Ericksen, 1983; McKay et al., 2003; Risacher et al., 2003; Clarke, 2006; Azua-Bustos et al., 2017). With its location in Chile, the desert is over 1000 km long and has a surface of about 105.000 square kilometres (Figure 1A). Due to the separation and resulting prevention of humidity by the Coastal Cordillera in the West and the rain shadow effect by the Andean Cordillera in the East (Figure 1B), it is a particularly isolated environment with stable arid conditions since about 150 million years (Jurassic period). This place turned hyper-arid during the Miocene period (Houston and Hartley, 2003; Hartley et al., 2005; Rech et al., 2006; Bull et al., 2018), or as other studies show, already in the Oligocene (Dunai et al., 2005). Additional factors are contributing to the hyper-aridity of the desert. Its location within the subtropical high-pressure belt where descending dry cold air (Hadley-circulation) creates an area with only little precipitation (Houston and Hartley, 2003) and the north-flowing Humboldt Current, with its cold upwelling and nutrient-rich water, prevents precipitation along South America's coast (Rundel et al., 1991; McKay et al., 2003; Hartley et al., 2005). Moisture generation occurs only at the coast through the formation of coastal fog (inversion layer, "camanchaca") or at higher altitudes (altiplano) via precipitation of rain or snow (Houston and Hartley, 2003; Cereceda et al., 2008). This precipitation creates a few perennial and ephemeral rivers crossing the Atacama Desert, mostly as groundwater below the surface (Houston, 2002, 2006). At first glance, barren landscape and aridity dominate the Atacama Desert. However, in the northern part of Chile, hypersaline inland waters (salars) are particularly common, and mainly located as a very few isolated

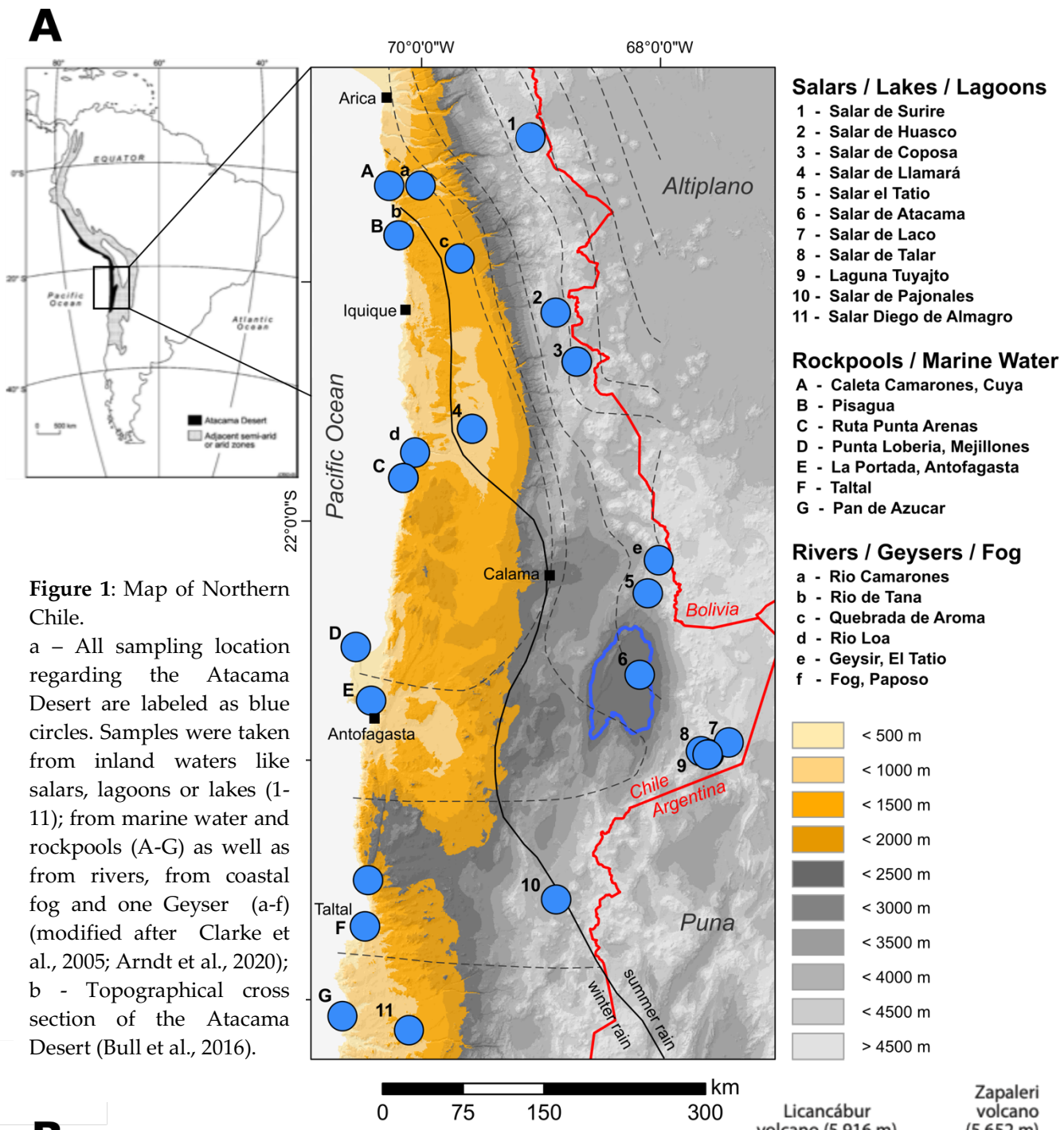

Figure 1: Map of Northern Chile.
a – All sampling location regarding the Atacama Desert are labeled as blue circles. Samples were taken from inland waters like salars, lagoons or lakes (1-11); from marine water and rockpools (A-G) as well as from rivers, from coastal fog and one Geyser (a-f) (modified after Clarke et al., 2005; Arndt et al., 2020); b - Topographical cross section of the Atacama Desert (Bull et al., 2016).

systems in the Central Depression (e.g. Salar de Llamará) and many in the Western Cordillera in the altiplano (e.g. Salar de Huasco, Salar de Coposa, ≥ 3800 m a.s.l.) (Risacher et al., 2003). The salars are permanent water bodies that receive water due to groundwater inflow from the East containing a relatively high amount of salt due to the leaching of volcanic rocks or due to seldom and infrequent flooding events (Demergasso et al., 2004). The salars located in the Pre-Cordilleran Depression (e.g. Salar de Atacama, Salar de Punta Negra) which are the oldest and largest in this area, gain water from the north, east and south. These inflows lead to a possible variation of salinity composition and concentration. The arid climate, the separation of the brines and the relatively low water supply, result in high evaporation rates, which leads to an increase in salinity within the system (Demergasso et al., 2004). This salinity can vary from subsaline to hypersaline conditions at different salars but also within one salar at lots of little pools the salinity can vary extremely (Demergasso et al., 2004). Hypersaline environments (salinity >50 practical salinity units (PSU)) develop from evaporation, either from seawater (thalassohaline environments) or from inland waters (athalassohaline environments), both with different ionic compositions (Oren, 2002; Voigt et al., 2020). These hypersaline environments are globally broadly but only rarely distributed (Javor, 1989). They occur in arid deserts as a result of high evaporation rates, in cold regions where freshwater removal of freezing water leads to an increase in salinity of the surrounding water and as well in the deep-sea, where geological salt deposits dissolve and hypersaline basins develop, e.g. Mediterranean deep-sea (Cita, 2006; Harding and Simpson, 2018). The Atacama Desert, together with its unique geological and hydrological conditions as an isolated habitat and the presence of hypersaline salt lakes within this region, makes it particularly interesting to study aquatic microbial diversity and adaptation. Life under these extreme conditions requires unique adaptations to the dryness and the high temperatures, high UV radiation, high and varying salt concentrations, and the

adaptation to high altitudes (in the altiplano). Due to their limited geographical distribution, their relatively simple food webs and the environmental pressure for their inhabitants, these hypersaline environments are especially interesting for research on microbial biogeography, ecology, and evolution with potentially highly adapted organisms and unique biodiversity (Warren, 2006; Harding and Simpson, 2018; Arndt et al., 2020).

Protists in hypersaline environments

Protists are unicellular and morphologically diverse organisms present in all different aquatic environments and soil as autotrophs, heterotrophs, mixotrophs as well as parasites or pathogens (Patterson, 1999; Dunlap, 2001; Guillou et al., 2008; Aurrecoechea et al., 2009; del Campo and Massana, 2011; Bates et al., 2013; Geisen et al., 2015). They occur in the entire eukaryotic tree of life (Figure 2C), and especially heterotrophic protists play an important role within microbial food webs as bacterial consumers and within various biogeochemical processes (Sherr and Sherr, 2002; Chambouvet et al., 2008; Adl et al., 2012; Geisen et al., 2018; Arndt et al., 2000). Due to their small size, the high reproduction rate and the ability to adapt quickly, they are potentially fast-evolving organisms (Lie et al., 2014; Arndt et al., 2020). The formation of cysts enables them to be transported into new habitats as well as to survive changing nonoptimal conditions e.g. the extremely high increase in salinity or habitats that temporary dry out (Patterson, 1999; Rogerson and Detwiler, 1999; Figueroa et al., 2011). Species living in hypersaline habitats, especially in salars within the Atacama Desert, have to cope with high environmental pressure like osmotic stress, along with turgor pressure and cellular dehydration (Rothschild and Mancinelli, 2001). In this case, they developed particular adaptations to overcome increasing osmotic pressure: The salt-in strategy, where organisms accumulate chloride- and potassium ions (Cl−and K+) intracellularly, or the salt-

out strategy, where salt ions are excluded from the cytoplasm, and at the same time high concentrations of compatible solutes are synthesized or accumulated. (Oren, 2002; Siglioccolo et al., 2011; Weinisch et al., 2018). In addition to the high salt concentration, the composition of various ions plays an important role. Especially in the Atacama Desert, potentially toxic heavy metals get into the aquatic systems through natural and industrial influences and provide an additional stress factor for the organisms (Salamanca et al., 2000; Lara et al., 2012; Volant et al., 2016). In previous studies on hypersaline environments, the diversity of prokaryotes was relatively well studied (Oren, 2002; Harding and Simpson, 2018; Dorador et al., 2020). However, only a few eukaryotic organisms have been reported from hypersaline water bodies, e.g. the algae *Dunaliella salina* as well as species of diatoms as primary producer, various ciliates like *Fabrea salina* or representatives of the genus *Euplotes* and the brine shrimp *Artemia salina* as main predator in these systems (Clavero et al., 2000; Pedrós-Alió et al., 2000; Oren, 2002; Triadó-Margarit and Casamayor, 2013; Harding and Simpson, 2018). Within these kinds of environments the species richness of eukaryotes decreases with rising salinities and is in general assumed to be low (Pedrós-Alió et al., 2000; Oren, 2002; Pedrós-Alió, 2004). Former studies, e.g., deep-sea research, showed that next-generation sequencing is an essential and reliable instrument in identifying previously unknown organisms (López-García et al., 2001; del Campo and Massana, 2011; de Vargas et al., 2015; Schoenle et al., 2021). Several recent studies on the molecular diversity of hypersaline environments detected a sizeable phylogenetic novelty within all taxonomic groups like archaea, bacteria, viruses, and eukaryotes (Demergasso et al., 2004; Dorador et al., 2013; Emerson et al., 2013; Triadó-Margarit and Casamayor, 2013; Oren, 2014; Eissler et al., 2019; Dorador et al., 2020; Lee et al., 2021). In eukaryotes, this novelty occurred especially within the supergroups of

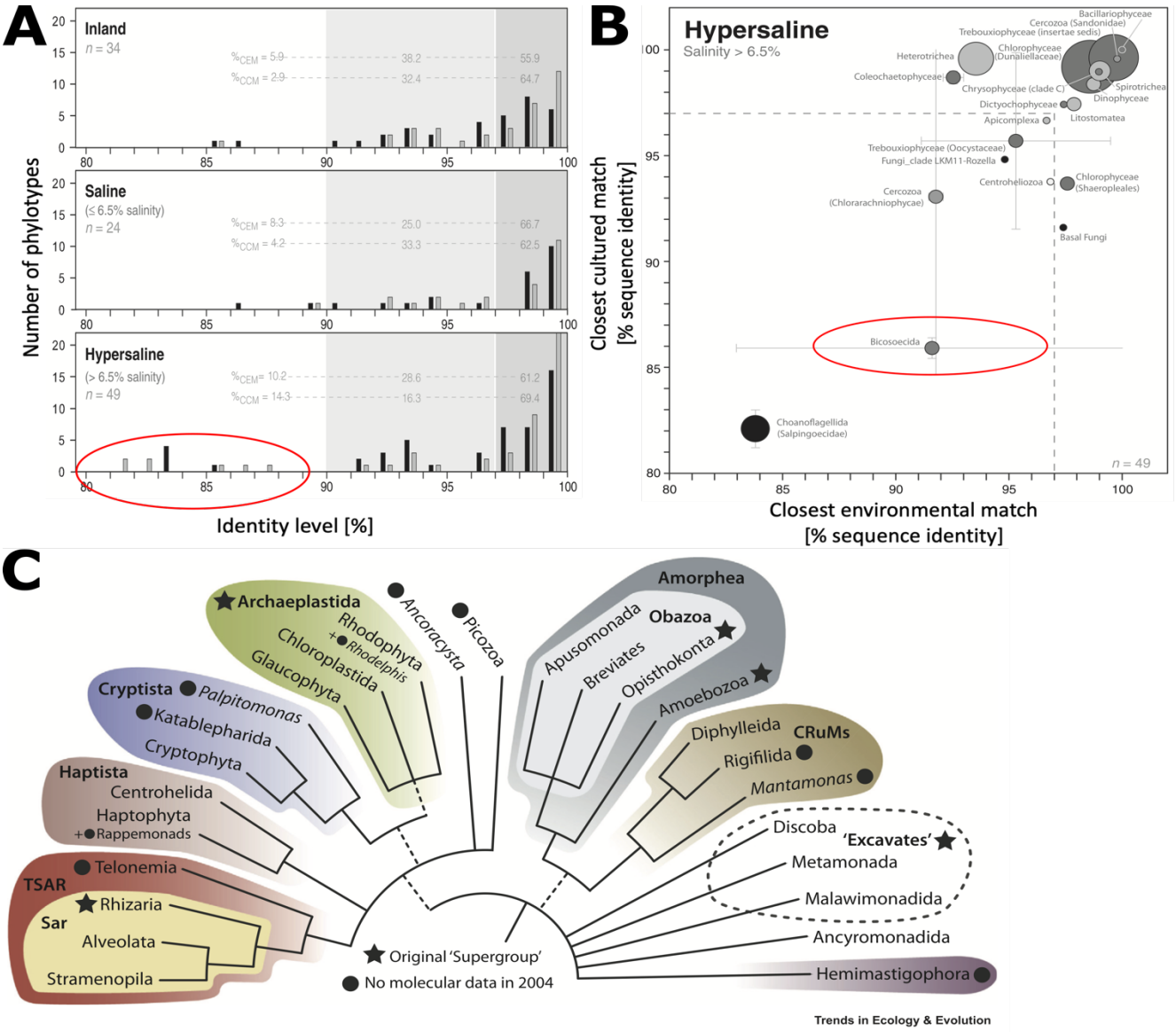

Figure 2: Genetic novelty and diversity in planktonic protists. A – High degree of phylogenetic novelty in inland, saline, and hypersaline environments. B – Protists to which this novelty can be assigned in hypersaline environments (adapted after (Triadó-Margarit and Casamayor, 2013). C - Phylogenetic tree of eukaryotes by Burki et al. (2020).

Stramenopiles (Bicosoecida) and Opisthokonta (Choanoflagellata), but many genotypes could not be assigned to any taxonomic group, halophile and halotolerant protists received much more attention and showed higher diversity than previously thought (Oren, 2002; Alexander et al., 2009; Triadó-Margarit and Casamayor, 2013; Zhao and Filker, 2018). Further species of Discoba (Heterolobosea) (Tikhonenkov et al., 2019) and Alveolata (Ciliophora) (Qu et al., 2020) from these hypersaline environments were described and

various studies on different taxa showed a special adaptation regarding their high range of salinity (Park and Simpson, 2010a; Schiwitza et al., 2018; Weinisch et al., 2018; Heine-Fuster et al., 2021). They also achieve their optimal growth at salinities three times higher than those in seawater (Park et al., 2006, 2007; Cho et al., 2008; Park and Simpson, 2010a; Foissner et al., 2014; Harding and Simpson, 2018). These results required additional investigations on the biodiversity and adaptation of protists in hypersaline environments (Harding and Simpson, 2018). Especially the recently discovered class Placididea within the supergroup of Stramenopiles was found in numerous different salars in the present study and turned out to be highly adapted to these environments and thus formed a focus within this study.

Placididea – Stramenopiles with high phylogenetic novelty

Stramenopiles are found in freshwater, brackish water, soil and marine habitats (Arndt et al., 2000). The entire biomass of heterotrophic flagellates within marine and freshwater systems consists of 20–50% of heterotrophic stramenopiles (Boenigk and Arndt, 2002) including common heterotrophic flagellate genera (e.g. *Bicosoeca*, *Cafeteria*, *Caecitellus*, *Developayella*, and *Pseudobodo*) and various uncultured marine stramenopiles (MASTs, MArine STramenopiles) (Massana et al., 2004; Massana and Pedrós-Alió, 2008). A further class of tiny heterotrophic stramenopiles, Placididea, was recently detected and represented only four described species, isolated from marine and brackish waters (Tong, 1997; Moriya et al., 2000, 2002; Park and Simpson, 2010a; Okamura and Kondo, 2015). *Pendulomonas adriperis* Tong, 1997 was the first morphologically described species and isolated from Southampton Water, U.K. (Tong, 1997). The description of *Wobblia lunata* Moriya et al., 2000 and *Placidia cafeteriopsis* Moriya et al., 2002, both isolated from Japanese coastal waters,

provided the first phylogenetic classification together with ultrastructural data, which also led to the establishment of the new class Placididea (Moriya et al., 2002, 2000). A new species was not described until 2015, when Yubuki et al. (2015) re-isolated and provided the first sequence of *Pendulomonas adriperis* from Canadian marine waters. *Suigetsumonas clinomigrationis* Okamura and Kondo 2015 was also described in this year from brackish waters in Japan (Okamura and Kondo, 2015). In 2010, first strains were isolated from mesosaline or hypersaline environments, but without a further morphological taxonomic description of these kinds of organisms (Luo et al., 2013; Park and Simpson, 2010a). They showed a particular adaptation to hypersaline conditions with a wide range of salinity tolerance (Park and Simpson, 2010b).

The evolution of protists

The distribution and evolution of metazoan or plant species was mainly investigated based on fossils. For some protists, there are paleontological records available, e.g. in diatoms (Kooistra and Medlin, 1996; Sims et al., 2006), Foraminifera (Culver, 1991; de Vargas et al., 1997; Pawlowski et al., 1997) and Polycystinea (Kunitomo et al., 2006). However, most protists contain no fossil evidence, making exact evolutionary dating difficult (Arndt et al., 2020; Parfrey et al., 2011). There are two different perspectives on how protists could be distributed. Due to the formation of cysts and their small size, it is assumed that they are globally ubiquitously dispersed via winds, birds, ocean currents and have only low allopatric speciation (Finlay and Clarke, 1999; Finlay and Fenchel, 1999; Finlay, 2002). On the other hand, there is the assumption that microorganisms are particularly adapted to their specific habitat (moderate endemicity model) (Foissner, 2006, 2007). The potential combination of geological- (e.g. formation of deserts and lakes) and genetic evolution of microorganisms in the Atacama Desert could make it possible to get an idea

about evolutionary processes and the distribution of heterotrophic protists. As an extreme and unique environment, the Atacama Desert, together with its isolated and extreme habitats populated by especially adapted aquatic organisms provides the best prerequisites to study the distribution and evolution of protists.

Aims

Within this thesis, the main objective was to enlarge the knowledge about protists' diversity, adaptation, community composition and distribution in extreme environments, illustrated by the Atacama Desert in the northern part of Chile. In this context, water from 24 different sites in the Atacama Desert was sampled to cover a wide range of different habitats (Figure 1A). A particular focus was placed on hypersaline inland waters (salars), which require special adaptations for their inhabitants due to their extreme conditions, especially the high salinity. Besides inland waters, lagoons, marine water, rivers, rockpools, fog and geysers, were sampled (Figure 3A-G). In addition to water samples used to isolate and cultivate protists, further water samples were taken to investigate the chemical composition. Water was also filtered at each location for metabarcoding analysis to sequence the V9 variable region of the 18S small subunit ribosomal DNA (SSrDNA) gene, to get more information about the community composition (Figure 3B). The RNA of placidid protists was isolated, and the transcriptomic data was used for further investigations on the evolution of protists. Furthermore, this data was also used to investigate the distribution of protists as part of the metabarcoding analysis (V9 SSrDNA gene of placidids).

All investigations within this thesis were summarized into three parts consisting of, in total, six chapters: Isolated and cultivated protists were investigated and described due to their morphology, ecology, and phylogenetical placement. We aimed to receive more information on the

protists living within these environments and how adapted to changing salinities they are (**Part 1**). Comparing different habitats within the metabarcoding analyses, we wanted to investigate the community composition and the distribution of certain protists as well as the possible exchange between different habitats (**Part 2**). Distinct protists were frequently isolated from hypersaline environments. The combination of molecular features of protists together with dated geological events were used to get more information about the speed of evolution of protists (**Part 3**).

The following hypotheses were addressed within the three parts:

- There is a high undescribed diversity within hypersaline environments
- There are protist groups that are especially adapted to hypersaline environments
- The salars are separated systems with only low possible exchange
- Each extreme aquatic system has its own unique protist community
- The evolution of protists correlates with the evolution of geological processes

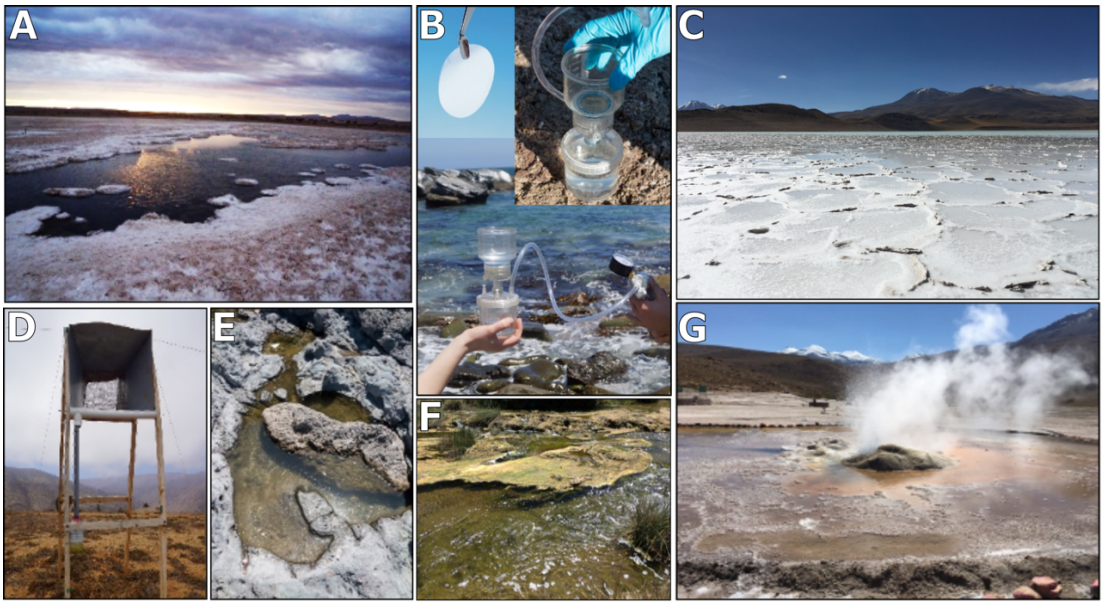

Figure 3: Different sampling locations in the northern part of Chile. A – Salar de Llamará; B – marine water and water filtration system for metabarcoding analysis; C – Laguna Tuyajto; D – Fog sampling system at Paposo; E – Rockpool; F – River system; G – Geyser.

Part 1

Halophilic organisms from extreme environments

Chapter 1

Revision of the phylogeny of Placididea (Stramenopiles): Molecular and morphological diversity of novel placidid protists from extreme aquatic environments. Investigations on hypersaline environments suggested that the microbial diversity of eukaryotes is widely underestimated. We frequently found representatives of heterotrophic Stramenopiles (Placididea) within these habitats. At the beginning of our study, only four species of placidids were isolated and fully described from marine and brackish waters. Within this study, we isolated 28 strains of placidids from mainly hypersaline inland waters located in the Atacama Desert (Chile), and from brackish and marine waters. Based on the combination of a multigene-based dataset, as well as morphological and ecological properties, we extended the phylogeny of the class of Placididea by three novel genera with nine new species. Placidids showed a separation into brackish-, marine- and athalassic clades with a wide range of salinity tolerance and a possible adaptation to extreme habitats. This study aimed to enlarge the under-investigated taxon of placidids and to describe and classify new species based on a combination of morphological, phylogenetical, and ecological features. *European Journal of Protistology, https://doi.org/10.1016/j.ejop.2021.125809.*

Chapter 2

Diversity and phylogeny of percolomonads based on newly discovered species from hypersaline and marine waters. Percolomonads were previously recorded mostly from marine and freshwater environments. Our studies

revealed, besides marine and brackish also strains from hypersaline inland waters located in the Atacama Desert. These strains turned out to be especially adapted to these extreme environments due to their high range of salinity tolerance. With this study, we wanted to resolve evolutionary relationships within the taxon of Percolomonadida and we described in this context three new genera and five new species based on their morphological, ecological, and phylogenetical characteristics. We provided further insights into especially adapted organisms isolated from extreme environments in the Atacama Desert and extended the under-investigated family which consisted of only two isolated and described species before. *European Journal of Protistology, https://doi.org/10.1016/j.ejop.2021.125808.*

Chapter 3

***Cafeteria* in extreme environments: description and ecological investigations on *C. burkhardae* and three new species (*C. baltica*, *C. biatacamiensis*, *C. paulosalfera*) from the Atacama Desert and the deep ocean.** Representatives of the genus *Cafeteria* were mainly isolated and described from marine habitats. Due to the isolation of ten strains from extreme environments (salars within the Atacama Desert and the deep sea), we aimed to enlarge the knowledge of the biodiversity and distribution of the genus *Cafeteria*. The combination of morphological, autecological, and phylogenetical investigations revealed the reisolation of *C. burkhardae* from coastal waters as well as the description of additional three new species. The representatives of *C. burkhardae* and *C. baltica* showed a wide range of salinity tolerance (up to 150 PSU), making it possible to populate different habitats with changing salinities. Two strains from the salars, isolated from the groundwater suggests a unique adaptation to relatively lower salt concentration.

Part 2

Biodiversity and adaptation of aquatic protists to extreme conditions in the Atacama Desert

Chapter 4

High diversity and isolated distribution of aquatic protists in salars of the Atacama Desert at extremely high salinities. With our metabarcoding analyses of 11 different inland waters located in the Atacama Desert, we aimed to take a deeper look into species composition and community structure of eukaryotes in hypersaline environments. It was possible to find unique eukaryotic communities at each location but also at different locations with potentially highly adapted organisms and only a small overlap between sampling sites. This suggests that only a small exchange between these separated systems takes place and allopatric speciation might have taken place. Mainly Stramenopiles and Alveolata turned out to be adaptable to variable changing salt concentrations which occur at different salars. Placidids were used as a model group of hypersaline environments. Especially the representatives of the genus *Allegra* that showed a wide range of salinity tolerance in our former investigations could be identified in this metabarcoding approach mainly in hypersaline environments.

Chapter 5

Biodiversity and distribution of protists in the Atacama Desert - metabarcoding analyses of various natural water transport systems in northern Chile. The transport of protists is possible due to the formation of cysts. Especially rivers and groundwater could possibly connect different salars and make an exchange between populations possible. Within this study, we investigated the protists' communities of 15 different water transport systems

like groundwater, rivers, fog, marine water, as well as one geyser in the northern part of Chile. Coastal rockpools were additionally investigated as potential microhabitats separated from the marine water with a recurring exchange. We wanted to get more information about possible transport mechanisms of protists and the possible exchange of different habitats. Placidids were used as a model group to investigate these distribution patterns. Mainly representatives of the genus *Allegra* could be identified in this metabarcoding approach, and we could show that they were distributed mainly through groundwater, fog and the geyser. Marine and rockpool species (from the genus *Wobblia*) showed to be unique to their marine systems. The samples were mainly dominated by Alveolata and Stramenopiles. Comparing various water transport systems as well as rockpools it was possible to find a mostly unique eukaryotic community at each location and only a small overlap between sampling sites. This suggests that only a small exchange of protists from other habitats via these transporting systems is taking place or that the species are especially adapted to their original habitat.

Part 3

The Evolution of Protists

Chapter 6

Mirroring the effect of geological evolution: Protist divergence in the Atacama Desert. We aimed to use the isolated extreme habitats in the Atacama as a model region to get more information on the evolution of protists. The combination of dated geological events together with molecular investigations provided fist insights into the divergence of protists in different extreme environments. Previous analysis, e.g. of placidids revealed a high diversity in these habitats. Molecular clock analysis using transcriptomic data showed

mutation rates correlating with the geological evolution of the salars located in the Atacama Desert. *Global and Planetary Change, doi: 10.1016/j.gloplacha.2020.103193.*

Part 1

Halophilic organisms from extreme environments

Chapter 1

Revision of the phylogeny of Placididea (Stramenopiles): Molecular and morphological diversity of novel placidid protists from extreme aquatic environment

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Revision of the phylogeny of Placididea (Stramenopiles): Molecular and morphological diversity of novel placidid protists from extreme aquatic environments

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Abstract

Recent studies suggested that the diversity of microbial eukaryotes in hypersaline environments is widely underestimated. Placidids are a group of heterotrophic stramenopile flagellates that are frequently found in these environments, but up to now only very few species were isolated and fully described, mostly from marine or brackish water sites. In this study, we extend the known diversity of Placididea by three new genera (*Allegra*, *Haloplacidia*, and *Placilonga*) compromising nine new species, isolated from athalassic, mostly hypersaline environments (*Allegra dunaii*, *Allegra atacamiensis*, *Allegra hypersalina*, *Haloplacidia cosmopolita, Suigetsumonas keniensis*) and marine waters (*Placilonga atlantica*, *Placidia azorensis*, *Placidia abyssalis, Wobblia pacifica*) including a description of their morphology and molecular phylogeny. In total, 36 strains were comparatively analysed. Studies from athalassic waters revealed an especially high number of different genotypes. A multigene analysis based on a ten genes dataset revealed a clear separation into marine, athalassic and brackish water clades. Several representatives were found to cope with hypersaline conditions from 20 to 250 PSU, even up to 284 PSU, suggesting that they may form a halotolerant group.

Keywords: Atacama Desert; Athalassic waters; Salinity tolerance; Extremophiles; Multigene analysis; Placididea

Introduction

Heterotrophic stramenopiles are major components, accounting for 20–50% of total heterotrophic flagellate biomass in the ocean and freshwater (Boenigk and Arndt 2002). This group includes common heterotrophic flagellates (e.g. *Bicosoeca*, *Cafeteria*, *Caecitellus*, *Developayella*, and *Pseudobodo*) and a diverse group of uncultured marine stramenopiles (MASTs, MArine STramenopiles) (Massana et al. 2004; Massana and Pedrós-Alió 2008). Placididea is a recently described class of small heterotrophic stramenopiles, with just four described species from marine

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and brackish waters (Moriya et al. 2002; Park and Simpson 2010; Okamura and Kondo 2015). *Pendulomonas adriperis* Tong, 1997, the first morphologically described species, was isolated from Southampton Water, U.K. (Tong 1997). Moriya et al. (2000, 2002) provided the first molecular and morphological descriptions of *Wobblia lunata* (Moriya et al., 2000), and *Placidia cafeteriopsis* Moriya et al., 2002, and established the class Placididea based on nuclear small subunit ribosomal DNA phylogenies and ultrastructural data from these species. Both species were isolated from Japanese coastal waters. Yubuki et al. (2015) re-isolated the morphospecies *Pendulomonas adriperis* from Canadian marine waters and provided the first sequence of this species. In the same year *Suigetsumonas clinomigrationis* Okamura and Kondo 2015, was isolated from brackish waters in Japan (Okamura and Kondo 2015). Additional placidids originating from Canada, Poland, South Africa, Kenya and Great Britain have had their 18S rDNA sequenced (Cavalier-Smith and Chao 2006; Park and Simpson 2010; Luo et al. 2013; Le Gall et al. 2015) but without formal taxonomic descriptions. The nuclear small subunit rDNA is widely used as a nuclear marker gene in many phylogenetic studies (Stoeck et al. 2010; Fonseca et al. 2014; Lie et al. 2014). Compared to other genes, it is highly conserved due to its slower evolution, e.g. mutation rate (Parfrey et al. 2011; Arndt et al. 2020). However, by using more than one gene for the eukaryote tree of life, the resolution of the tree could be greatly improved (Burki et al. 2020). In recent years, multigene analyses have been used successfully to resolve the phylogeny within several protist groups (Brown et al. 2013; Pánek et al. 2015; Carr et al. 2017; Lax et al. 2021).

Hypersaline environments (usually greater than 40 PSU) originate either from evaporation of seawater (thalassohaline environments) or from evaporation of inland waters (athalassohaline environments). The ionic composition of both hypersaline environments may differ greatly (Voigt et al. 2020). Life under these extreme conditions requires particular adaptations, for example organisms have to cope with osmotic stress along with cellular dehydration (e.g. Rothschild and Mancinelli 2001; Weinisch et al. 2018). Nevertheless, both thalassohaline and athalassohaline waters are populated by a large variety of different microbes that have adapted to sometimes very high salinities (up to salt saturation conditions). While the diversity of prokaryotes is relatively well studied, little is known on the diversity of microbial eukaryotes (e.g. Oren 2002; Harding and Simpson 2018; Dorador et al. 2020). Investigations of the molecular diversity of microbial eukaryotes in hypersaline waters revealed a large number of novel genotypes which could not be assigned to any taxonomic group (Triadó-Margarit and Casamayor 2013; Zhao and Filker 2018), for review see Harding and Simpson (2018). Recently, new choanoflagellate genera from hypersaline waters with distinct morphologies and broad salinity tolerances were described (Schiwitza et al. 2018). *Enibas tolerabilis* grows at freshwater up to hypersaline conditions (70 PSU) a previously undescribed property for acanthoecid choanoflagellates (Schiwitza et al. 2019).

By analysing many different marine, thalassic and athalassic hypersaline waters, especially from the Atacama Desert (Northern Chile), we discovered a large diversity of placidids. We isolated and cultivated 28 new strains. By combining studies on morphology and ecology with a multigene analysis we enlarge the class by four genera and eight new species, and provide an extended placidid phylogeny.

Material and methods

Sampling and cultivation

Samples of surface water from northern Chile were collected from various hypersaline lakes and coastal rock pools during several expeditions. Further samples were taken from Lake Nakuru (surface water), Kenya, the Atlantic Ocean and the Caribbean Sea (benthic multicorer samples, expedition M139 with R/V Meteor) and from deep saline groundwater of a hard coal mine in Germany (Tab. 1, Fig. S1). For comparison, already isolated and described Placididea from previous studies were also included into this study: Two strains each were isolated from a salt seep on Salt Spring Island in Canada, from the Wieliczka Salt Mine in Poland (Park and Simpson 2010) as well as *Suigetsumonas clinomigrationis* which was isolated from the brackish meromictic Lake Suigetsu (2–14 PSU) in Japan (Kondo et al. 2006; Okamura and Kondo 2015). Two strains of *Placidia cafeteriopsis* (NIES-1013; NIES-1014) and one strain of *Wobblia lunata* (NIES-1015) (Moriya et al. 2000, 2002) were ordered from the NIES Collection - Microbial Culture Collection in Japan. All three strains originate from enrichment cultures of coastal waters of Japan.

All cultures were inoculated into 50 ml-tissue culture vessels (Sarstedt, Nümbrecht, Germany) filled with 30–35 ml medium containing artificial hypersaline water (AHW, 35PSU (l^{-1}): 35.256 g NaCl, 0.886 g KCl, 4.43 g $MgCl_2 \cdot 6H_2O$, 0.21 g MgSO4x7H_2O, 0.151 g $CaCl_2$x2H_2O with 10% of a cereal grass stock solution as a carbon source for autochthonous bacteria. The salt concentration was adjusted close to the salt concentration of the sampling site. For the cereal grass stock solution, 1 g cereal grass (Lebepur GmbH, Berlin, Germany) was added to 1 L of AHW medium with the required salinity, autoclaved and subsequently filtered.

To obtain new clonal cultures, dilutions based on the liquid aliquot method were performed (Butler and Rogerson 1995) from raw cultures (AHW medium), or cells were isolated using a micromanipulator (Patchman MP2, Eppendorf, Hamburg, Germany). For initial cultivation the salinity of

the AHW medium was adjusted to the salinity of the original habitat. For long-term cultivation, cultures were stepwise transferred into 35 PSU AHW medium.

Important parameters of all strains used in this study, such as the exact geographic location, strain designation, and concentration of source salinity are shown in Table 1.

RNA isolation, sequencing and bioinformatic analyses

In total, 20 out of 36 strains of Placididea were selected for RNA isolation (Table 1). All strains were inoculated into multiple flasks containing 40 ml AHW medium (35 PSU) together with cereal grass, except for HFCC 1221, 1412 and 1413, which were cultivated at salinities closer to the original sampling site salinities to enhance growth (100-, 5-, 100 PSU). RNA isolation was carried out using the peqGOLD Total RNA Kit (peqlab (VWR, Erlangen, Germany) at a density of approx. 10^6 cells/ml in each culture flask: Ten culture flasks per strain were scraped and the medium transferred to 50 ml tubes (Sarstedt, Nümbrecht, Germany) and centrifuged with 3000 *g* at 10 °C for 15 min. The supernatant was discarded, 400 µl lysis buffer were added to the first tube, vortexed and subsequently passed from one tube to another to obtain enough cells at low buffer volume, all steps were performed at short time and at ice to preserve RNA. The following steps were performed after manufacturer protocol, except for an additional washing step. The quality of each sample was checked via Nanodrop ND-1000 (Peqlab, VWR, Erlangen, Germany) and stored afterwards at −80 °C.

RNA samples were sequenced after a Poly(A)-selection via Illumina HiSeq 3000 (Illumina, San Diego, California) (paired-end, 2x75 bp) at the Cologne Center of Genomics (CCG). Biased sequences and reads with lengths below 36 base pairs and/or ambiguities were eliminated from the dataset via Trimmomatic-0.32 (Bolger et al. 2014). Cologne High Efficiency Operating Platform for Science (CHEOPS) was used to perform the assembling by using Trinity 2.0.6 (Grabherr et al. 2011). Contigs were blasted against a reference gene database to search for candidate genes. All contigs were checked for chimeras using UCHIME (Edgar et al. 2011) by the use of the command chimera.uchime in all sequences. No chimeras were detected.

DNA isolation, sequencing and analyses

The amplification of the 18S- and 28S rDNA was performed by DNA extraction and standard polymerase chain reaction (PCR). 40 ml of clonal strains were transferred into 50 ml tubes (Sarstedt, Nümbrecht, Germany) and centrifuged with 4000 *g* at 4 °C for 20 min. The supernatant was discarded, and DNA was extracted using the Quick gDNA ™ Mini Prep Kit (Zymo Research Corporation, CA, USA) applying the manufacturers' protocol. 3 µl of the isolated DNA was used as a template together with 5 µl of each universal primer with a final concentration of 1 µM as well as a Taq DNA Polymerase Master Mix (2x) (VWR CHEMICALS, Haasrode, Belgium; final volume of 50 µl). The amplification of the 18S rDNA with the primer pairs 18S For (5′ -AACCTGGTTGATCCTGCCAGT- 3′, Medlin et al. 1988) and 18S Rev (5′ -TGATCCTTCCGC AGGTTCACCTAC- 3′, Medlin et al. 1988) started with the denaturation step at 96 °C for 2 min, then 34 cycles of 96 °C for 30 s, 55 °C for 45 s, and 72 °C for 2.5 min, then a final elongation step for 7 min at 72 °C. To amplify the partial LSU the primer pairs NLF184/21 (5′ -ACCCGCTGA AYTTAAGCATAT- 3′) and NLR2098/24 (5′ -AGCCA ATCCTTWTCCCGAAGTTAC- 3′) or NLF1105/22 (5′ -C CGAAGTTTCCCTCAGGATAGC- 3′) and NLR2098/24 (Van der Auwera et al. 1994) were used with the denaturation temperature of 98 °C for 2 min, then 35 cycles of 98 °C for 30 s, two annealing steps of 55 °C for 45 s and 52 °C for 2.5 min, and an elongation step at 72 °C for 2.5 min, then a final elongation step for 10 min at 72 °C. The PCR products were verified by gel electrophoresis using a 1% agarose gel. PCR products were purified afterwards using the Bioscience PCR Purification Kit (Bioscience, Jena, Germany) and sequenced by using the 18S rDNA and 28S rDNA primer sets at GATC Biotech, Germany. Sequences were analyzed using NCBI nucleotide BLAST® (Altschul et al. 1990; Wheeler and Bhagwat 2007).

Phylogenetic analysis

Sequences for ten genes (18S- and 28S rDNA, ITS1, 5.8S, ITS2, α-tubulin, β-tubulin, EF1-α and EF2, hsp90), which have been proven to be suitable for phylogenetic investigations in protists (Parfrey et al. 2011), were extracted from the assembled transcriptome data and reference data from GenBank, if available, was downloaded. Each gene was aligned separately using ClustalX 2.1 (Larkin et al. 2007) for quality control to ensure the correct, homologous contig was selected, followed by checking and manual correction in BioEdit V7.0.0 (Hall 1999). All genes were joined into a concatenated dataset resulting in an alignment of 15913 bp. Nucleotide sequences from protein-coding genes were additionally translated into protein sequences, using ExPASy (web.expasy.org/translate/) and aligned to verify the functionality of those genes. All genes were treated equally (unpartioned model) with regard to the rate of evolution. The model used for the inference of phylogenetic trees was determined with MrAIC (Nylander 2004). The Maximum Likelihood (ML) tree was calculated using RAxML-HPC BlackBox version 8.2.12 (Stamatakis 2014) on CIPRES Gateway (Miller et al. 2010) with the GTR + FO + I + G model. The calculations for the Bayesian inference (BI) trees were carried out using MrBayes v3.2.1 (Ronquist and Huelsenbeck 2003) with a GTR + I + Γ model and a four-category gamma distribution for 1,000,000 generations and a 25% burn-in. The trees were

Table 1. List of Placididea strains used in this study including the location, the salinity and the altitude at the sampling site of the source population, the corresponding accession numbers in GenBank, and the reference in literature. Strains used for RNA isolation are marked with an asterisk. Underlined names of isolates refer to new species. HFCC - Heterotrophic Culture Collection Cologne, PSU - Practical Salinity Units.

Species	Isolate	Geographic location	Salinity [PSU] / Altitude [m]	18S	ITS1 5.8S ITS2	28S	Reference
Placilonga atlantica	HFCC1505*	Seawater originating from the West Atlantic Ocean, coast of Florida	15	MW591529			this study
Placidia azorensis	HFCC952	**Atlantic Ocean,** Azores - Flores Island 39°23.376′N, 30°36.721′W	35 / −1400	MW575545	–	–	this study
Placidia abyssalis	HFCC739*	**Caribbean Sea,** Deep Sea - R/V Meteor M139 15°53′13.44″N, 68°55′44.58″W	35 / −4033	MW575540	–	MW774210	this study
	HFCC745*	**Caribbean Sea,** Deep Sea - R/V Meteor M139 15°53′13.44″N, 68°55′44.58″W	35 / −4033	MW575541	–	MW774211	this study
Wobblia pacifica	HFCC834*	**Chile - Coastal Rockpools,** Pisagua 19°35′50″S, 70°12′52″W	93 / 1	MW591530			this study
	HFCC5000	**Chile - Coastal Rockpools,** Caleta Punta Arenas 21°38′ 27′'S, 70°08′ 43′'W	125 / 2	MW575546	–	–	this study
Allegra dunaii	HFCC1221*	**Chile - Salar de Atacama** 23°17′19.7″S, 68°10′37.8″W	138 / 2301	MW591531			this study
	HFCC1252*	**Chile - Salar de Talar** 23°55′7.49″S, 67°41′50.56″W	28 / 3930	MW591532			this study
	ME8*	**Poland - Wieliczka Salt Mine** 49°59′0.75″N, 20°3′11.97″E	63	GU170208	–	–	Park & Simpson (2010)
				MW676122		MW774212	this study
	HFCC1258*	**Chile - Salar de Surire** 18°47′42.40″S, 69°5′7.69″W	154 / 4245	MW591533			this study
	HFCC1242	**Chile - Salar de Surire** 18°47′42.40″S, 69°5′7.69″W	154 / 4245	MW575547	–	MW774217	this study
	HFCC5009	**Chile - Salar de Coposa** 20°40′41′'S 68°42′09′'W	40 / 3729	MW575548	–	–	this study
	HFCC1261*	**Chile - Salar de Surire** 18°47′42.40″S, 69°5′7.69″W	154 / 4245	MW591534			this study
Allegra atacamiensis	HFCC5011	**Chile - Salar de Llamará** 21°16′07″S, 69°37′01″W	142 / 738	MW575549	–	–	this study
	HFCC1400	**Chile - Salar de Llamará** 21°16′15.62″S, 69°37′8.78″W	128 / 800	MW575550	–	MW774218	this study
Allegra hypersalina	HFCC5015	**Chile - Salar de Llamará** 21°16′07″S, 69°37′01″W	149 / 738	MW575551	–	–	this study
	HFCC5021	**Chile - Salar de Llamará** 21°16′07″S, 69°37′01″W	152 / 738	MW575552	–	MW774219	this study
	HFCC1413*	**Chile - Salar de Llamará** 21°16′15.62″S, 69°37′8.78″W	128 / 800	MW591535			this study
Suigetsumonas keniensis	HFCC1412*	**Kenya - Lake Nakuru** 0°21′22.20″S, 36°6′0.76″E	5	MW575542	–	MW774213	this study

Haloplacidia cosmopolita	HFCC207*	**Germany - Ibbenbüren,** Hard Coal Mine 52°17′14.35″N, 7°43′53.60″E	100 / −1346	MW591536			this study
	HFCC1408	**Chile - Salar de Atacama** 23°17′18.2″S, 68°10′35.8″W	132 / 2301	MW575553	–	MW774220	this study
	ME5*	**Poland - Wieliczka Salt Mine** 49°59′0.75″N, 20° 3′11.97″E	73	MW591537			Park & Simpson (2010) this study
	HFCC1222	**Chile - Salar de Atacama** 23°17′18.2″S, 68°10′35.8″W	132 / 2301	MW575554	–	MW774221	this study
	HFCC881	**Chile - Salar de Huasco** 20°18′20.6″S, 68°53′5.5″W	150 / 3780	MW575555	–	–	this study
	HFCC209	**Germany - Ibbenbüren,** Hard Coal Mine 52°17′14.35″N, 7°43′53.60″E	70 / −940	MW575556	–	MW774222	this study
	MESS13*	**Canada - Salt Spring Island** 48°46′0.20″N, 123°27′19.36″W	65	GU170212 MW675781	–	MW774214	Park & Simpson (2010) this study
	HFCC1211*	**Chile - Salar de Atacama** 23°17′18.2″S, 68°10′35.8″W	132 / 2301	MW591538			this study
	HFCC1407*	**Chile - Salar de Llamará** 21°16′06.6″S, 69°37′00.7″W	50 / 742	MW591539			this study
	HFCC1409*	**Chile - Salar de Atacama** 23°17′16.6″S, 68°10′26.6″W	114 / 2301	MW591540			this study
	HFCC1247	**Chile - Salar de Atacama** 23°19′38.79′’S, 68°09′58.69′’W	102 / 2301	MW575557	–	MW774223	this study
	HFCC1410	**Chile - Salar de Atacama** 23°17′22.3″S, 68°10′42.1″W	132 / 2301	MW575558	–	–	this study
	MESS14	**Canada - Salt Spring Island** 48°46′0.20″N, 123°27′19.36″W	65	GU170213	–	MW774224	Park & Simpson (2010) this study
ALREADY PUBLISHED SPECIES							
Wobblia lunata	NIES-1015*	**Japan,** Osabe Harbor Rikuzen-Takada, Kesencho, Iwate 39° 0′0.68″N, 141°37′47.29″E	35	AB032606 MW575543	– –	– MW774215	Moriya et al. (2000) this study
Placidia cafeteriopsis	NIES-1013*	**Japan,** Tokyo Bay Yokohama, Kanagawa 35°27′15.87″N, 139°39′16.22″E	35	MW591541			Moriya et al. (2000) this study
	NIES-1014	**Japan,** Kamaishi Harbor Kamaishi, Minatocho, Iwate 39°15′28.80″N, 141°55′15.60″E	35	– MW575559	–	–	Moriya et al. (2000) this study
Suigetsumonas clinomigrationis	NIES-3647*	**Japan,** Lake Suigetsu, Fukui 35°35′5.31″N, 135°52′15.97″E	15 / −7	AB976561 MW575544	– –	– MW774216	Okamura & Kondo (2015) this study

sampled every 100 generations until the value for the average standard deviation of split frequencies reached 0.01. For phylogenetic analysis, transcriptome sequences showed differences to already published strains. The sequences from strains GU170207, GU170208 and GU170212; GU170213 from Canada and Poland were corrected in this study and in case of *Placidia cafeteriopsis* (NIES-1013, NIES-1014), *Wobblia lunata* (NIES-1015) and *Suigetsumonas clinomigrationis* (NIES-3647) transcriptome data of the present study were additionally added to the tree.

Light microscopy

Morphological structures were investigated as described by Jeuck et al. (2014). In brief, the cultures were observed with an inverted light microscope by Zeiss with an Axio Observer, a 100x/1.4NA oil immersion objective (DIC) and a water immersion condenser. The cultures were observed in Petri dishes with coverslip bases. High-resolution video microscopic records were obtained using a black/white analogous Hamamatsu C6489 camera with noise suppression and contrast amplification by an Allen Video-Enhanced Contrast (AVEC) system (Hamamatsu, Argus-20) (Brabender et al. 2012). The video records for each strain were evaluated frame-by-frame with VirtualDub (www.virtualdub.org) and subsequently processed with ImageJ (Abràmoff et al. 2004; Table S1). Morphological characteristics (cell length, cell width, length of flagella, nucleus diameter) of specimens were measured with Axio Vision Rel. 4.8 (Zeiss, Germany; Table S1). Comparisons of each morphological feature for the different species was carried out with ANOVA and Tukey´s post-hoc test using RStudio (RStudio Team, 2015. Integrated Development Environment for R, Boston, MA; http://www.rstudio.com; Version 4.0.2).

Electron microscopy

Cultures were fixed with 2.5% glutaraldehyde in 0.05 M cacodylate buffer (final concentration) for one hour at 4 °C, then filtered using a polycarbonate track-etched membrane filter (Whatman™, Nuclepore™, 2 µm pore size). Filters were dehydrated by an ethanol series of 30 to 96%. Filters were washed twice at each concentration and remained for 10 min in the fluid. Finally, a 50:50 Hexamethyldisilazane (HMDS)-ethanol solution was added for 10 min and two washing steps with pure HMDS and 5 min incubation time were performed. Filters were air-dried, stuck to a sample holder and coated with a 120 Å layer of gold (Nitsche and Arndt 2008). The samples were investigated using scanning electron microscopy (SEM) (FEI Quanta 250 FEG).

Experiments on salinity tolerance

One ml of each culture in exponential growth phase was inoculated into Petri dishes filled with 20 ml AHW medium (35 PSU) and cereal grass. Three replicates each were used to increase salinity (50 PSU first, then in 25 PSU steps up to 225 PSU) and decrease salinity (first 25 PSU followed by 15 PSU, 10 PSU, 4 PSU, 2 PSU) every 48 h after checking for living cells. All strains were exposed to each salinity for seven days at 20 °C until the respective limit was reached for salinity (high/low) where no living cells could be observed. In addition, the last salinity step was repeated by adding additionally heat-killed bacteria to the cultures (*Pseudomonas putida* MM1) in order to exclude food limitation.

ZooBank registration

ZooBank registration number of the present study: urn:lsid:zoobank.org:pub:BE888B6D-350C-41A8-910D-92A3F77B3D2B.

Results

Morphological investigations

In total, 28 novel clonal strains of Placididea were isolated within this study (Table 1). All strains were studied using molecular methods, nine of those were additionally analysed regarding their morphological identity. In general, placidids were round to kidney- or D-shaped and 3–10 µm long by 2–7 µm wide (Fig. 1A, M, W, AC). The anterior part of the cell is elongated and includes a feeding structure (Fig. 1F, N, P, AL, AM). The placidids bear two flagella of different length that are directed anteriorly (min.-max. = 4–13 µm) and posteriorly (2–10 µm) (Fig. 1O, R, V, W, Z, AD, AF, AN). Both flagella emerge 0.5–1 µm subapically from a shallow groove on the ventral side of the cell (Fig. 1O, Z, AD). The posterior flagellum is smooth (Fig. 3-A-I), i.e. no structures are visible, and used to attach to the substrate, while the anterior flagellum bears mastigonemes (Fig. 3A-F, H-I) and shows a fast whip-like beating, resulting in a trembling movement of attached cells. This behaviour is differently pronounced in different species (videos SV01-09). The studied placidids were most frequently attached to the substrate (Fig. 1D, H, O), some strains were also found to glide or swim (Fig. 1AC). Swimming cells have the anterior flagellum anterograde and slightly rotate during swimming. The position of the nucleus was generally close to the bases of flagella (Fig. 1C, U, Q, AJ). Roundish to ovoid resting stages (cysts) were observed under stress conditions (e.g. high salinity, low food supply) at least for three species (Fig. 2).

Phylogenetic analysis

The highly supported concatenated tree of ten genes obtained by transcriptome sequencing provided a well-resolved phylogeny for Placididea. Calculated p-distances

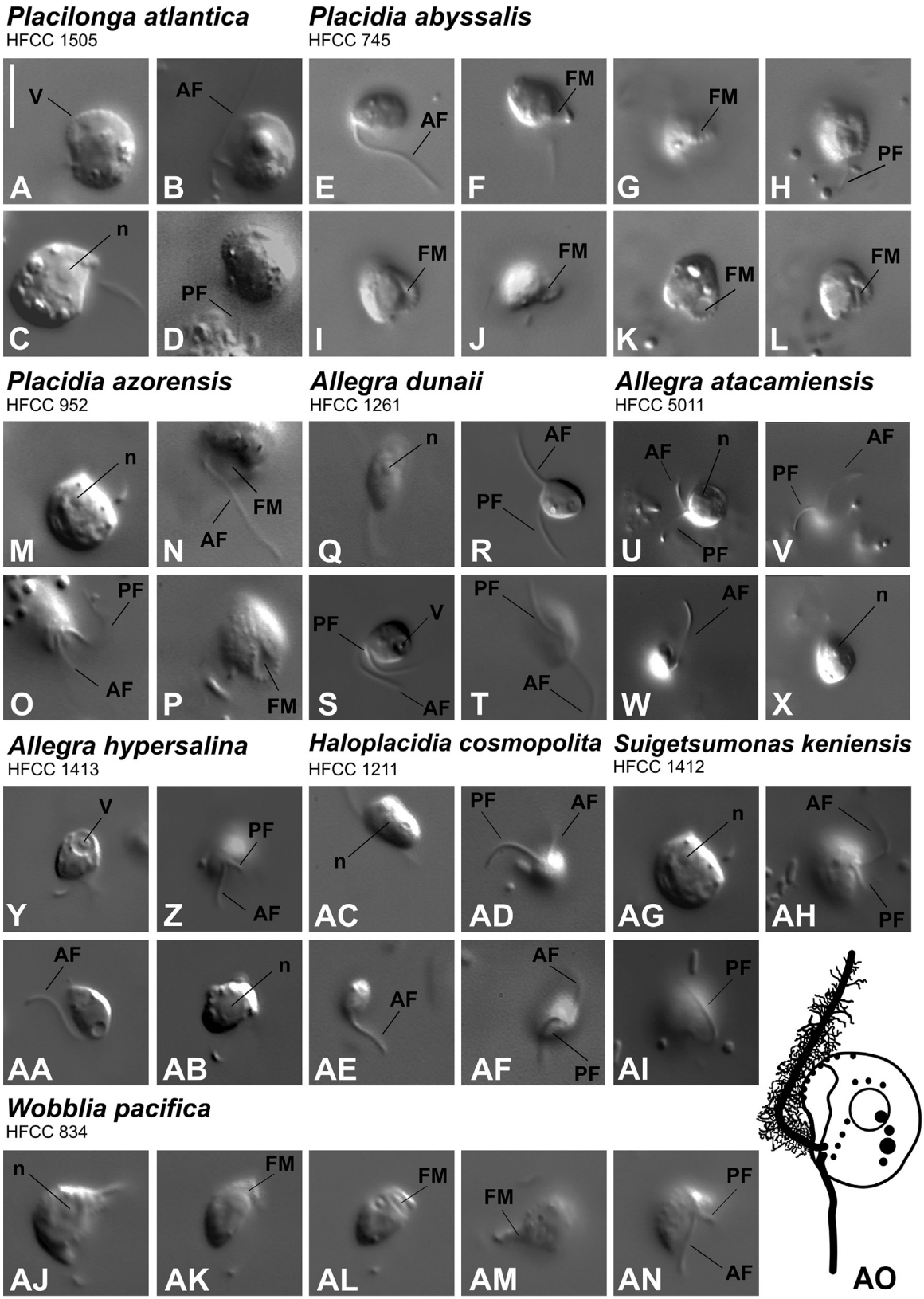

Fig. 1. Morphological characteristics of newly described Placididea. A-D: *Placilonga atlantica* sp. nov. (HFCC 1505); E-L: *Placidia abyssalis* sp. nov. (HFCC 745); M−P: *Placidia azorensis* sp. nov. (HFCC 952); Q-T: *Allegra dunaii* sp. nov. (HFCC 1261); U-X: *Allegra atacamiensis* sp. nov. (HFCC 5011); Y-AB: *Allegra hypersalina* sp. nov. (HFCC 1413); AC-AF: *Haloplacidia cosmopolita* sp. nov. (HFCC 1211); AG-AI: *Suigetsumonas keniensis* sp. nov. (HFCC 1412); AJ-AN: *Wobblia pacifica* sp. nov. (HFCC 834); AO: Drawing of *Placilonga atlantica* sp. nov. (HFCC 1505). Scale bar of 5 μm in image A applies to all light microscopic images. AF - anterior flagellum; PF - posterior flagellum; v - vacuole; n - nucleus.

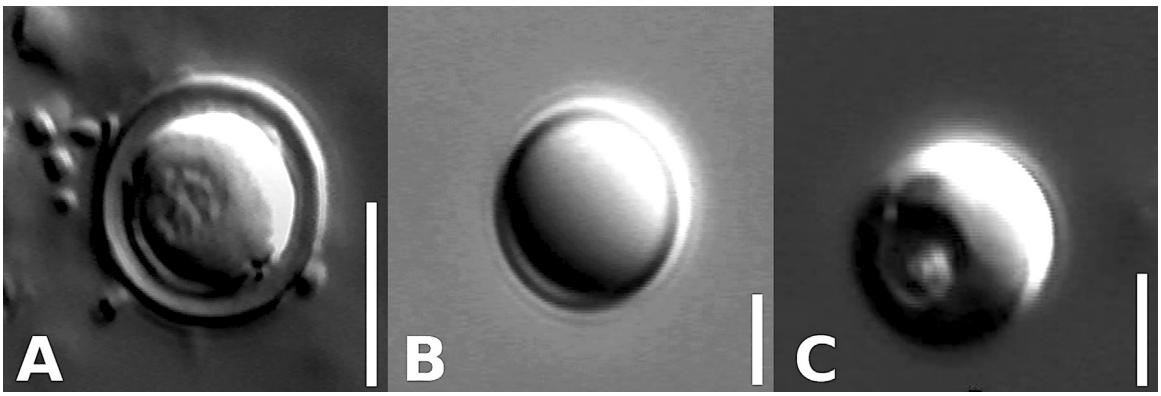

Fig. 2. Light-microscopic images of placidid resting stages (cysts). A: Strain HFCC 1400; B: *Placidia azorensis* sp. nov. (HFCC 952); C: HFCC1222. Scale bars in A and B represent 5 μm, in C 2 μm.

based on pairwise comparisons of the uncorrected 18S rDNA sequences indicated the clear separation of taxa (Tab. S2). The strains from the Atlantic Ocean (HFCC 1505, HFCC 952) clustered together with the strains from the Caribbean Sea (HFCC 739, HFCC 745) and *Placidia cafeteriopsis* from Japan. Based on the phylogenetic position of the strain HFCC 1505, which branched basally within this clade (p-distance of 18S rDNA to HFCC 952 = 12.6%), a new species and genus (*Placilonga atlantica* gen. et. sp. nov.) was established. The other strain from the Atlantic Ocean, *Placidia azorensis* sp. nov. (HFCC 952), clustered within the clade *Placidia* but formed a basal branch with a p-distance of 18S rDNA = 2.4% to *Placidia cafeteriopsis*. The other strains within this clade, originating from the Caribbean Sea (*Placidia abyssalis* sp. nov., HFCC 745 and HFCC 739), showed a p-distance of 18S rDNA of 1.1% to *Placidia cafeteriopsis*. Resequencing the strain NIES-1014 (one of the two *Placidia cafeteriopsis* strains from the Microbial Culture Collection NIES) showed small differences compared to the data deposited at NCBI. According to Cavalier-Smith and Chao (2006), the previously published 18S rDNA included a fungal contaminant and was therefore left out of our analysis. The previously isolated strains GU170207, GU170208 and GU170212, GU170213 from Canada and Poland (Park and Simpson 2010) were also resequenced in this study and the corrected sequences were used in the present analysis. The strains HHCC 745 and HFCC 739 did not show differences in the 18S rDNA, α-tubulin, β-tubulin, EF1-α, EF2 and hsp90. In this case, both strains were considered to belong to the same species, *Placidia abyssalis* sp. nov., with HFCC 745 as the type strain. The type strain *Wobblia pacifica* sp. nov., (HFCC 834) isolated from a Chilean rockpool in Pisagua was closely related to the strain HFCC 5000 isolated from a rock pool about 300 km to the South. *Wobblia pacifica* sp. nov., (HFCC 834) clustered together with *Wobblia lunata* (p-distance of 18S rDNA = 1.0%) from Japan. Strain HFCC 5000 showed no difference to the 18S rDNA of *Wobblia pacifica* sp. nov., however, the transcriptome was not sequenced and thus no further comparison was possible. The strain *Allegra dunaii* sp. nov. (HFCC 1261) was isolated from Salar de Surire in the Atacama. Other phylogenetically related strains were isolated from different salars in the Atacama Desert in Chile (see Table 1) (HFCC 1221, 1252, 1258, 1242, and 5009) as well as from a salt mine in Poland (ME8). All these strains form a cluster. Most closely related to *Allegra dunaii* sp. nov. (HFCC 1261) are strains from the Salar de Llamará: *Allegra atacamiensis* gen. et sp. nov. (HFCC 5011, p-distance of 18S rDNA to *Allegra dunaii* = 4.7%.), and *Allegra hypersalina* sp. nov. (HFCC 1413, p-distance of 18S rDNA to *Allegra dunaii* = 4.1%). *Suigetsumonas keniensis* sp. nov. (HFCC 1412) isolated from the Lake Nakuru in Kenya is related to *Suigetsumonas clinomigrationis* (p-distance of 18S rDNA = 4.0%) from Japan. The last clade comprised 13 closely related strains, the strain HFCC 1211 isolated from the Salar de Llamará was assigned as the type strain for *Haloplacidia cosmopolita* gen. et sp. nov. Seven additional strains from different salars in the Atacama as well as five strains from Poland, Germany and Canada clustered together. Within the 'species-level' clades listed above most strains showed slight molecular differences from the type strain for the species. For this reason, we refer to them by their strain designations instead of assigning them to these species (e.g. Fig. 4), with the exception of the *Placidia abyssalis*; strain HFCC 739 (mentioned above).

Salinity tolerance

Representatives of all athalassic genera (*Allegra, Haloplacidia)* showed the highest salinity tolerance of up to 250 PSU. *Allegra atacamiensis* and *Allegra hypersalina* were unable to tolerate salinities lower than 20 PSU, whereas, *Allegra dunaii* and *Haloplacidia* also tolerated lower salinities (4 PSU). The thalassic hypersaline species *Wobblia pacifica* was only able to survive at salinities from 15 to 75 PSU. The species isolated from marine waters (*Placilonga atlantica, Placidia azorensis, Placidia abyssa-*

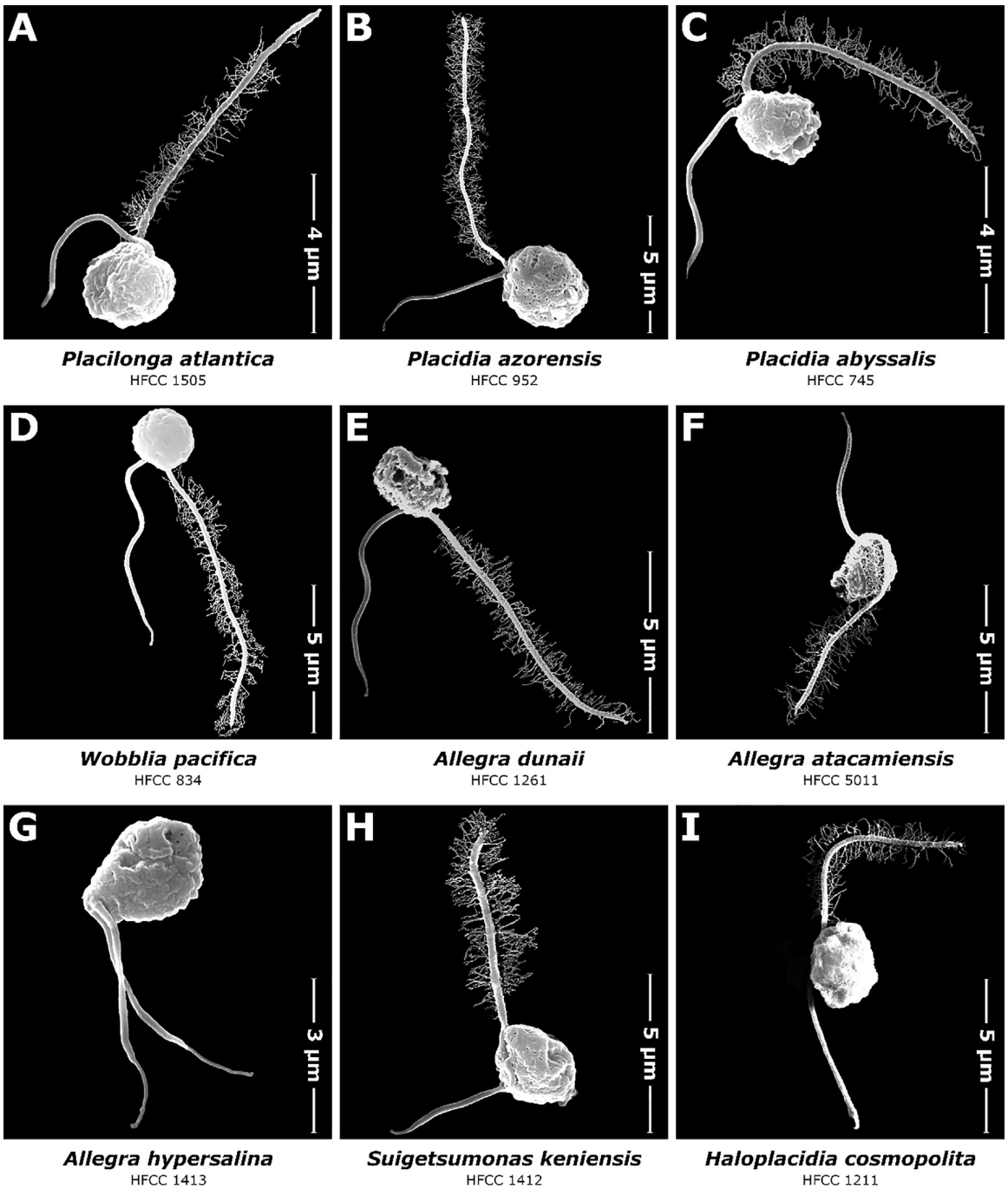

Fig. 3. Scanning electron-microscopical images of Placididea. A: *Placilonga atlantica* sp. nov. (HFCC 1505); B: *Placidia azorensis* sp. nov. (HFCC 952); C: *Placidia abyssalis* sp. nov. (HFCC 745); D: *Wobblia pacifica* sp. nov. (HFCC 834); E: *Allegra dunaii* sp. nov. (HFCC 1261); F: *Allegra atacamiensis* sp. nov. (HFCC 5011); G: *Allegra hypersalina* sp. nov. (HFCC 1413); H: *Suigetsumonas keniensis* sp. nov. (HFCC 1412); I: *Haloplacidia cosmopolita* sp. nov. (HFCC 1211). Scale bars in B, D, E, F, H and I represent 5 µm, in A and C 4 µm and in G 3 µm, respectively.

lis) showed similar salinity tolerances (from 15 to 75 PSU) except for *Wobblia lunata,* which tolerated salinities from 4 to 130 PSU. The brackish water isolates of the genus *Suigetsumonas* were the only placidids, that also tolerated salinities of 0 PSU (up to 84 PSU; Fig. 5).

Taxonomic summary

Stramenopiles Patterson 1989, emend. Adl et al. 2005
Bigyra Cavalier-Smith 1997, emend. 2006
Placidida Moriya et al. 2002

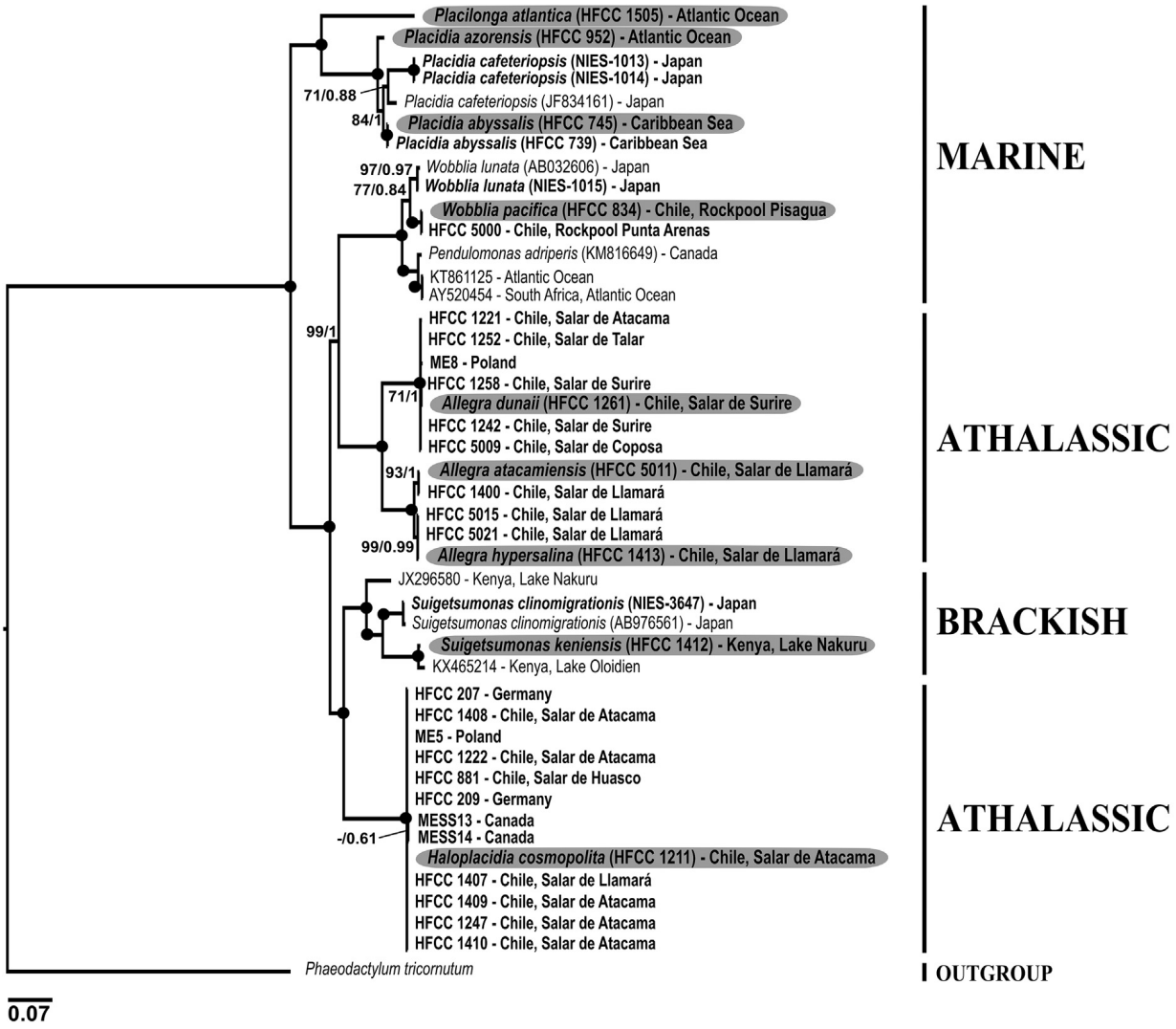

Fig. 4. Phylogenetic tree of Placididea based on ten concatenated genes: 18S- and 28S rDNA, ITS1, 5.8S, ITS2, α-tubulin, β-tubulin, EF1-α, Ef2 and hsp90. ML bootstrap percentages (first value) and Bayesian posterior probabilities (PP: second value) are given for each node. Supporting values < 50% or 0.5 are labelled with a dash (-). Solid circles indicate 100% bootstrap support and PP = 1.

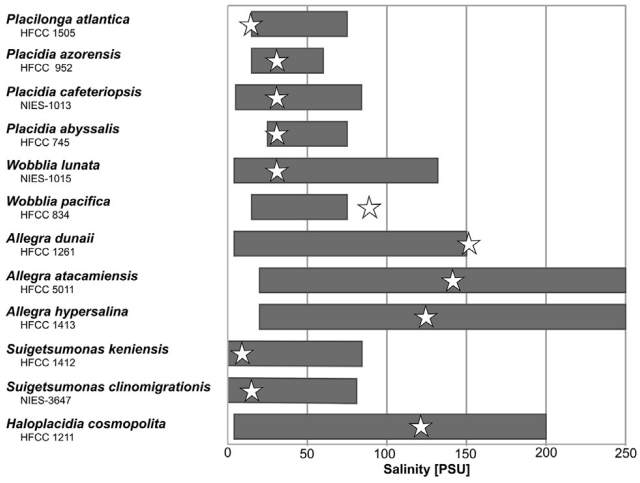

Fig. 5. Salinity tolerance ranges of all described Placididea species. Stars indicate the original salinity of the source material.

Placididae Moriya et al. 2002

Placilonga gen. nov. Rybarski & Arndt

Description: Kidney-shaped to round organisms, uninucleate with two flagella, posterior flagellum smooth, conspicuously long anterior flagellum with flagellar hairs (mastigonemes). Frequently attached to the substrate via posterior flagellum, anterior flagellum beating, phagotrophic feeding, brackish to marine environment. Forms a distinct phylogenetic branch separate from the most closely related genus, *Placidia.*

Etymology: *placidus* = Latin word for quiet or peaceful referring to the cells being attachment to the substrate; *longa* = Latin word for long, describing the conspicuously long anterior flagellum (name, feminine).

ZooBank registration: urn:lsid:zoobank.org:act:537E1015-3331-4F45-BEBE-51C477EB40AD.

Type species: *Placilonga atlantica* Rybarski & Arndt, this publication

Placilonga atlantica sp. nov. Rybarski & Arndt; Fig. 1 (A-D); type strain: Heterotrophic Flagellate Collection Cologne HFCC 1505.

Description: Cells kidney-shaped (Fig. 1A), length 4.2 – 7.6 µm (min. - max.; mean ± SD = 5.7 ± 0.9 µm, n = 20), width 3.1 – 6.4 µm (4.6 ± 1.1 µm, n = 20). Two flagella, long anterior flagellum, length 3.8 – 12.8 µm (8.3 ± 2.5 µm, n = 20), shorter posterior flagellum, characterized by mastigonemes (Fig. 3A), length 3.9 – 9.3 µm (6.6 ± 1.6 µm, n = 20) (Fig. 1B, D). Both flagella emerge from a shallow groove at ventral side of the cell. Nucleus located close to this groove (Fig. 1C), diameter 0.9 – 1.8 µm (1.3 ± 0.3 µm, n = 10). Cells frequently attached to substrate by posterior flagellum (Fig. 1D), anterior flagellum oriented in the opposite direction (Fig. 1B) creates feeding current for bacterial uptake. Anterior part of the cell slightly elongated, forming a feeding opening. Swimming cells usually not observed. Representatives of this species tolerate salinity from 15 to 75 PSU (Fig. 5).

Etymology: *atlantica* referring to its origin, the Atlantic Ocean (adjective).

Type material: An electron microscopy preparation of HFCC 1505 has been deposited in the Biology Centre of the Museum of Natural History in Upper Austria, Linz, number EVAR 2021/11. This material constitutes the name-bearing type (an hapantotype) of the species.

ZooBank registration: urn:lsid:zoobank.org:act:4B8BF4BB-1D8E-4D0A-9047-BE45D17C9B96.

Type locality: Shrimp recirculation aquaculture system at the University of Cologne inoculated with water originating from the West Atlantic Ocean, coast of Florida.

Type sequence: Sequences are deposited in the GenBank database: 18S rDNA to 28S rDNA: MW591529. Further genes and their accession numbers can be found in the supplementary material (Table S3).

Remarks: The closest related described species are *Placidia cafeteriopsis*, isolated from Japanese marine waters (p-distance of 18S rDNA = 12.9%; JF834161) and *Placidia abyssalis*, described within this study and isolated from the abyssal zone of the Caribbean Sea (p-distance of 18S rDNA = 12.3%).

Comparing with *Placidia* species, cells of *Placilonga atlantica* are larger (average length × width = 6 × 5 µm) than *Placidia abyssalis* (average length × width = 5 × 4 µm) and shorter than *Placidia azorensis* (average length × width = 7 × 5 µm) and *Placidia cafeteriopsis* (average length × width = 7 × 5 µm). Comparing with all other placidids, just *Suigetsumonas clinomigrationis* bears an anterior flagellum of similar maximum length (13 µm); all other placidids bear shorter anterior flagella. Cells show relatively slow movement and no rapid trembling movement like e.g. *Wobblia* species.

Placidia Moriya et al. 2002

Placidia azorensis sp. nov. Rybarski & Arndt; Fig. 1 (M−P); type strain: Heterotrophic Flagellate Collection Cologne HFCC 952.

Description: Ovoid to elliptic or irregularly shaped cells, length 5.5 – 8.6 µm (min. - max.; mean ± SD = 6.7 ± 1.0 µm, n = 20), width 4.0 – 7.3 µm (5.3 ± 0.8 µm, n = 20). Two flagella, long anterior flagellum (Fig. 1N), length 6.7 – 12.0 µm (9.1 ± 1.3 µm, n = 20), shorter posterior flagellum (Fig. 1O), length 4.1 – 8.2 µm (5.7 ± 1.1 µm, n = 20). Both flagella emerge from a shallow groove at the ventral side of the cell (Fig. 1O). Nucleus located close to this groove (Fig. 1M), diameter 0.7 – 1.1 µm (1.0 ± 0.1 µm, n = 20). Anterior part of the cell elongated, forming a feeding opening (Fig. 1N, P). Cells attached to the substrate by posterior flagellum. Anterior flagellum characterized by mastigonemes (Fig. 3B), oriented in the opposite direction, creating a feeding current for bacterial uptake (Fig. 1O). Swimming cells not observed. Salinity tolerance in a range of 10 to 60 PSU (Fig. 5). Cysts present, roundish to ovoid, length 3.0 – 7.6 µm (4.3 ± 1.1 µm, n = 20), width 3.4–8.4 µm (4.7 ± 1.2 µm, n = 20) (Fig. 2B).

Etymology: *azorensis,* referring to the origin of isolation, the Azores in the Atlantic Ocean.

Type material: An electron microscopy preparation of HFCC 952 has been deposited in the Biology Centre of the Museum of Natural History in Upper Austria, Linz, number EVAR 2021/12. This material constitutes the name-bearing type (a hapantotype) of the species.

ZooBank registration: urn:lsid:zoobank.org:act:647378DE-2B25-43A9-81B2-80A976136125.

Type locality: Atlantic Ocean, Island Flores, Azores (39°23.376′N, 30°36.721′W), at a salinity of 35 PSU.

Type sequence: The 18S rDNA (MW575545) sequence is deposited in the GenBank database.

Remarks: The closest related described species are *Placidia cafeteriopsis*, isolated from Japanese marine waters (p-distance of 18S rDNA = 2.4%; JF834161) and *Placidia abyssalis*, described within this study and isolated from the abyssal zone of the Caribbean Sea (p-distance of 18S rDNA = 1.7%). In culture, the cell moves rather slowly compared to the almost trembling movement of other placidids. The cells of *Placidia azorensis* are larger (average of length × width = 7 × 5 µm) than those of the closely related Placidia abyssalis (average of length × width = 5 × 4 µm); also, the anterior flagellum (average of: anterior flagellum = 9 µm, posterior flagellum = 6 µm) is slightly longer than that of *P. abyssalis* (average of: anterior flagellum = 8 µm, posterior flagellum = 8 µm). Within the genus *Placidia*, *P. azorensis* has a similar cell- length and width to *P. cafeteriopsis* (average of length × width = 7 × 5 µm), Comparing with other placidids, *P. azorensis* is similar in length to other placidids isolated from marine waters (*Wobblia pacifica, Pendulomonas adriperis*). Cells show relatively slow movement and no rapid trembling movement like e.g. *Wobblia* species.

Placidia abyssalis sp. nov. Rybarski & Arndt; Fig. 1 (E-L); type strain: Heterotrophic Flagellate Collection Cologne HFCC 745.

Description: Kidney-shaped cells (Fig. 1E), length 3.8 – 5.9 µm (min. - max.; mean ± SD = 4.6 ± 0.5 µm, n = 20), width of 2.3 – 4.3 µm (3.5 ± 0.5 µm, n = 20). Two flagella, long anterior flagellum (Fig. 1E), length of 4.7 – 10.8 µm (7.6 ± 1.7 µm, n = 20), shorter posterior flagellum (Fig. 1H), length of 3.1 – 10.1 µm (5.4 ± 1.7 µm, n = 20). Both flagella emerge from a shallow groove at the ventral side of the cell. Cells are attached to the substrate by the smooth posterior flagellum, moving only a little from the spot by gliding with the posterior flagellum, while the cell body has a slow wobbling motion. The mastigoneme-bearing anterior flagellum (Fig. 3C) oriented in the opposite direction creating a feeding current for bacterial uptake. When bacteria get attached to the flagellum, *Placidia abyssalis* pushes them actively into feeding opening. (Fig. 1I-L). Representatives of this species tolerate salinity from 25 to 75 PSU (Fig. 5).

Etymology: *abyssalis*, referring to its origin from the abyssal of the Caribbean Sea.

Type material: An electron microscopy preparation of HFCC 745 has been deposited in the Biology Centre of the Museum of Natural History in Upper Austria, Linz, number EVAR 2021/13. This material constitutes the name-bearing type (an hapantotype) of the species.

ZooBank registration: urn:lsid:zoobank.org:act:CE31FDF0-31CD-46DD-9435-177FD1AE34D0.

Type locality: Caribbean Sea, Deep Sea (4033 m; 15° 53′13.44″N, 68°55′44.58″W) at a salinity of 35 PSU.

Type sequence: All sequences are deposited in the GenBank database: 18S rDNA: MW575541, 28S rDNA: MW774211. Further genes and their accession numbers can be found in the supplementary material (Table S3).

Remarks: The closest related described species is *Placidia cafeteriopsis*, isolated from Japanese marine waters (p-distance of 18S rDNA = 1.5%; JF834161).

Placidia abyssalis is a relatively small placidid and is smaller (average length × width = 5 × 4 µm) than the closely related *Placidia cafeteriopsis* (average length × width = 7 × 5 µm). Despite being the smallest member of genus *Placidia, P. abyssalis* bears an anterior flagellum of nearly equal length (average length = 8 µm) to that of the other species (average length = 8–9 µm). A similarly long posterior flagellum (maximum length = 10 µm) was only recorded for *Placidia abyssalis*, *Wobblia pacifica* and *Suigetsumonas clinomigrationis*. Detached cells swim in a circle until they sink down and attach again to the substrate. Attached cells show relatively slow movement and no rapid trembling movement like e.g. *Wobblia* species.

Wobblia Moriya et al. 2000

Wobblia pacifica sp. nov. Rybarski, Filz, Nitsche & Arndt; Fig. 1 (AJ-AN); type strain: Heterotrophic Flagellate Collection Cologne HFCC 834.

Description: D-shaped cells (Fig. 1AJ), length of 5.3 – 7.7 µm (min. - max.; mean ± SD = 6.7 ± 0.6 µm, n = 20), width of 3.1 – 4.7 µm (4.0 ± 0.5 µm, n = 20). Two flagella, long fast beating anterior flagellum, acronematic posterior flagellum, length of 4.6 – 10.0 µm (7.6 ± 1.5 µm, n = 20) (Fig. 1AN). Both flagella emerge from a shallow groove at the ventral side of the cell (Fig. 1AN). Nucleus located close to this groove (Fig. 1AJ), diameter of 1.0 – 1.4 µm (1.2 ± 0.1 µm, n = 20). Anterior part of the cell elongated (Fig. 1AM) forming a feeding opening. Cells frequently attached to the substrate by posterior flagellum, swimming cells present. The mastigoneme-bearing anterior flagellum (Fig. 3D) is oriented in the opposite direction and creates a feeding current for bacterial uptake into the feeding opening. Representatives of this species tolerate salinity from 25 to 75 PSU (Fig. 5). Cysts present when the salinity reached higher concentrations.

Etymology: *pacifica*, referring to its origin, the Pacific Ocean (adjective).

Type material: An electron microscopy preparation of HFCC 834 has been deposited in the Biology Centre of the Museum of Natural History in Upper Austria, Linz, number EVAR 2021/14. This material constitutes the name-bearing type (an hapantotype) of the species.

ZooBank registration: urn:lsid:zoobank.org:act:43DB6DB4-A3F4-4221-B243-77DFFB2A72D5.

Type locality: Costal rock pool in Pisagua, Northern Chile (19°35′50″S, 70°12′52″W) at salinity of 93 PSU.

Type sequence: All sequences are deposited in the GenBank database: 18S rDNA to 28S rDNA: MW591530. Further genes and their accession numbers can be found in the supplementary material (Table S3).

Remarks: The closest related described species is *Wobblia lunata*, isolated from Japanese marine waters (p-distance of 18S rDNA = 1.0%; AB032606). *Wobblia pacifica* is slightly smaller (average length × width = 7 × 4 µm) than *W. lunata* (average length × width = 8 × 5 µm) and is similar in size to *Pendulomonas adriperis*, which is the closest known relative to the genus *Wobblia*. The movement of *Wobblia* cells and the anterior flagellum show the fastest trembling motion within the placidids described up to now. In this respect *W. pacifica* and *W. lunata* do not differ from each other.

Allegra gen. nov. Rybarski & Arndt

Description: Small-sized, roundish till ovoid-shaped organism, uninucleate, two flagella, posterior flagellum smooth, anterior flagellum equipped with flagellar hairs (mastigonemes). Usually attached to the substrate via posterior flagellum, anterior flagellum mostly beating very fast, swimming cells move in anterior direction with the anterior flagellum anterograde, anterior part of the cell is slightly elongated, forming a feeding opening. Cells have a very fast trembling movement, phagotrophic feeding. Form resting stages (roundish cysts) in case of suboptimal ecological conditions.

Etymology: *Allegra* = From the Italian word for "lively" and referring to the fast movement (noun, feminine).

ZooBank registration: urn:lsid:zoobank.org:act:F00B701B-B79F-45A8-B7A8-9758C39BF656.

Type species: *Allegra atacamiensis* Rybarski & Arndt, this publication

Allegra dunaii sp. nov. Rybarski, Nitsche & Arndt; Fig. 1 (Q-T); type strain: Heterotrophic Flagellate Collection Cologne HFCC 1261.

Description: Roundish till ovoid-shaped organism (Fig. 1Q), length of 3.9–6.9 μm (min. - max.; mean ± SD = 5.1 ± 0.7 μm, n = 20), width of 2.7–4.6 μm (3.4 ± 0.5 μm, n = 20). Two flagella, long anterior flagellum, length of 4.7–9.3 μm (6.7 ± 1.4 μm, n = 20), a shorter posterior flagellum, length of 4.3–8.3 μm (6.2 ± 1.0 μm, n = 20) (Fig. 1R). Both flagella emerge from a shallow groove at the ventral side of the cell (Fig. 1T). Nucleus located close to this groove (Fig. 1Q), diameter of 0.4–0.9 μm (0.6 ± 0.1, n = 20). Cells frequently attached to the substrate by posterior flagellum, anterior mastigoneme-bearing flagellum (Fig. 3E) oriented in the opposite direction creating a feeding current for bacterial uptake. Swimming cells move in anterior direction with the anterior flagellum in anterograde position. The cell and the anterior flagellum have a very fast trembling movement. Representatives of this species tolerate salinity from 4 to 150 PSU (Fig. 5). Roundish to ovoid cysts present at salinities higher than 150 PSU or other unfavourable ecological conditions.

Etymology: *dunaii,* dedicated to Tibor Dunai for his engagement in fostering geological and biological studies in the isolated hypersaline environments of the Atacama (name, masculine).

Type material: An electron microscopy preparation of HFCC 1261 has been deposited in the Biology Centre of the Museum of Natural History in Upper Austria, Linz, number EVAR 2021/15. This material constitutes the name-bearing type (an hapantotype) of the species.

ZooBank registration: urn:lsid:zoobank.org:act:44798156-C590-42B9-A49D-7401BDD1E967.

Type locality: Salar de Surire (18°47′42.40″S, 69°5′7.69″W) a hypersaline lake in northern Chile at an altitude of 4245 m at a salinity of 154 PSU.

Type sequence: All sequences are deposited in the GenBank database: 18S rDNA to 28S rDNA: MW591534. Further genes and their accession numbers can be found in the supplementary material (Table S3).

Remarks: The closest related isolated strain is ME8, from Wieliczka Salt Mine, Poland (p-distance of 18S rDNA = 0.2%; GU170208) and Allegra hypersalina, described within this study and isolated from the Salar de Llamará (128 PSU) in Northern Chile (p-distance of 18S rDNA = 4.1%). Allegra dunaii bears an anterior and posterior flagellum relatively similar in length (average length 6.7 and 6.2 μm, respectively). The phylogenetically distant Haloplacidia cosmopolita is the only other placidid showing this character. Cells show a moderately rapid trembling movement faster than Placidia and Placilonga and slower than Wobblia species.

Allegra atacamiensis sp. nov. Rybarski, Schmidt, Nitsche & Arndt; Fig. 1 (U-X); type strain: Heterotrophic Flagellate Collection Cologne HFCC 5011.

Description: Roundish to ovoid-shaped organism (Fig. 1U, W, X), length of 3.5 – 5.8 μm (min. - max.; mean ± SD = 4.3 ± 0.6 μm, n = 20), width of 2.1 – 3.5 μm (2.9 ± 0.3 μm, n = 20). Two flagella, long anterior flagellum (Fig. 1V-W), length of 6.9 – 12.0 μm (8.8 ± 1.2 μm, n = 20), a shorter posterior flagellum (Fig. 1U-V), length of 3.0 – 6.5 μm (4.2 ± 1.0 μm, n = 20). Both flagella emerge from a shallow groove at the ventral side of the cell (Fig. 1V). Nucleus located close to this groove (Fig. 1U, X), diameter of 0.5–1.1 μm (0.8 ± 0.1, n = 20). Anterior part of the cell slightly elongated (Fig. 1X) forming a feeding opening. Cells are trembling very fast, frequently attached to the substrate by posterior flagellum, swimming cells observed. The mastigoneme-bearing anterior flagellum (Fig. 3F) is oriented in the opposite direction and creates a feeding current for bacterial uptake into the feeding opening. Salinity tolerance in a range of 20 to 284 PSU (Fig. 5). Cysts present when the salinity reached concentrations higher than 284 PSU or in conditions of starvation (Fig. 2A).

Etymology: *atacamiensis,* referring to its origin, Salar de Llamará in the Atacama Desert, Chile.

Type material: An electron microscopy preparation of HFCC 5011 has been deposited in the Biology Centre of the Museum of Natural History in Upper Austria, Linz, number EVAR 2021/16. This material constitutes the name-bearing type (an hapantotype) of the species.

ZooBank registration: urn:lsid:zoobank.org:act:1887E63C-B424-4D08-A44C-949E360FF805.

Type locality: Salar de Llamará (21°16′07″S, 69°37′01″W), a hypersaline lake in northern Chile at an altitude of 738 m, at salinity of 149 PSU.

Type sequence: The 18S rDNA (MW575549) sequence is deposited in the GenBank database.

Remarks: The closest related isolated strain is ME8, from Wieliczka Salt Mine, Poland (p-distance of 18S rDNA = 4.7%; GU170208) and Allegra hypersalina, described within this study and isolated from the Salar de Llamará (128 PSU) in Northern Chile (p-distance of 18S rDNA = 1.5%). Allegra atacamiensis belongs among the smaller placidids from hypersaline environments. The cells of Allegra atacamiensis are slightly larger (average length × width = 5 × 4 μm) than those of Allegra hypersalina (average length × width = 4 × 3 μm), but the flagella are of similar length (average of: anterior flagellum = 7 μm, posterior flagellum = 5 μm). Cells show a very fast trembling movement, but slower than in Wobblia species.

Allegra hypersalina sp. nov. Rybarski, Nitsche & Arndt; Fig. 1 (Y-AB); type strain: Heterotrophic Flagellate Collection Cologne HFCC 1413.

Description: Roundish to ovoid-shaped organism (Fig. 1AB), length of 3.4–5.2 µm (min. - max.; mean ± SD = 4.3 ± 0.6 µm, n = 20), width of 2.2–4.1 µm (3.1 ± 0.6 µm, n = 20). Two flagella emerge from a shallow groove at the ventral side of the cell (Fig. 1Z), long anterior flagellum, length of 4.5 – 10.7 µm (6.7 ± 1.3 µm, n = 20), slightly shorter posterior flagellum with a length of 3.2 – 7.6 µm (5.3 ± 1.0 µm, n = 20) (Fig. 1Z). The anterior flagellum does not bear mastigonemes (Fig. 3G). Nucleus located close to this groove (Fig. 1AB), diameter of 0.7 – 1.2 µm (0.9 ± 0.1 µm, n = 20). Anterior part of the cell slightly elongated (Fig. 1AA) forming a feeding opening. Cells are trembling very rapidly, frequently attached to the substrate by posterior flagellum, swimming was observed. Anterior flagellum oriented in the opposite direction creating a feeding current for bacterial uptake into the feeding mouth. Representatives of this species tolerate salinity ranging from 20 to 250 PSU (Fig. 5).

Etymology: *hypersalina,* referring to the hypersaline environment the strain was isolated from (adjective).

Type material: An electron microscopy preparation of HFCC 1413 has been deposited in the Biology Centre of the Museum of Natural History in Upper Austria, Linz, number EVAR 2021/17. This material constitutes the name-bearing type (an hapantotype) of the species.

ZooBank registration: urn:lsid:zoobank.org:act:FC554711-F707-4524-810A-FC5F6190A10C.

Type locality: Salar de Llamará (21°16′15.62″S, 69°37′8.78″W), a hypersaline lake in Northern Chile at an altitude of 800 m at a salinity of 128 PSU.

Type sequence: All sequences are deposited in the GenBank database: 18S rDNA to 28S rDNA: MW591535. Further genes and their accession numbers can be found in the supplementary material (Table S3).

Remarks: The closest related isolated strain is ME8, from Wieliczka Salt Mine, Poland (p-distance of 18S rDNA = 4.2%; GU170208) and Allegra atacamiensis, described within this study and isolated from the Salar de Llamará (128 PSU) in Northern Chile (p-distance of 18S rDNA = 1.6%). Allegra hypersalina is one of the smaller placidids from hypersaline environments. The cells of A. hypersalina are slightly smaller (average length × width = 4 × 3 µm) than those of A. atacamiensis (average length × width = 5 × 4 µm). The flagella of both Allegra species are of a similar size (average: anterior flagellum = 7 µm, posterior flagellum = 5 µm), but the anterior flagellum of A. hypersalina does not bear mastigonemes. Cells show a moderately rapid trembling movement, but much slower than in Wobblia species.

Suigetsumonas Okamura & Kondo 2015

Suigetsumonas keniensis sp. nov. Rybarski & Arndt; Fig. 1 (AG-AI); type strain: Heterotrophic Flagellate Collection Cologne HFCC 1412.

Description: Kidney-shaped cells (Fig. 1AG), length of 3.6 – 6.7 µm (min. - max.; mean ± SD = 5.2 ± 0.8 µm, n = 20), width of 2.6 – 5.3 µm (3.9 ± 1.0 µm, n = 20). Two flagella that emerge from a shallow groove at the ventral side of the cell (Fig. 1AH): long anterior flagellum (Fig. 1AH) and a shorter posterior flagellum (Fig. 1AI), length of 4.4 – 8.0 µm (mean ± SD = 6.3 ± 1.1 µm, n = 20). Nucleus located close to this groove (Fig. 1AG), diameter of 0.6 – 1.2 µm (mean ± SD = 1.0 ± 0.2 µm, n = 17). The anterior part of the cell is slightly elongated and forms a feeding opening. Cells are not trembling rapidly, frequently attached to the substrate by posterior flagellum, the mastigoneme-bearing anterior flagellum (Fig. 3H) is oriented in the opposite direction creating a feeding current for bacterial uptake. Swimming cells observed, in case of detachment sink down slowly and then immediately re-attach to the substrate by posterior flagellum. Representatives of this species tolerate salinity from 0 to 84 PSU (Fig. 5).

Etymology: *keniensis*, dedicated to the sampling site in Kenya (Lake Nakuru) and the people living in this region.

Type material: An electron microscopy preparation of HFCC 1412 has been deposited in the Biology Centre of the Museum of Natural History in Upper Austria, Linz, number EVAR 2021/18. This material constitutes the name-bearing type (an hapantotype) of the species.

ZooBank registration: urn:lsid:zoobank.org:act:F1C79BB5-7DF2-4CE8-83DF-082533FE15AF.

Type locality: Lake Nakuru, Kenya (0°21′22.20″S, 36°6′0.76″E) at a salinity of 5 PSU.

Type sequence: All sequences are deposited in GenBank database: 18S rDNA: MW575542, 28S rDNA: MW774213. Further genes and their accession numbers can be found in the supplementary material (Table S3).

Remarks: The closest related strain is the uncultured stramenopile clone OL65, from Lake Oloidien, Kenya (p-distance of 18S rDNA = 0.6%; KX465214) and the closest related described species is Suigetsumonas clinomigrationis, isolated from Lake Suigetsu in Japan (p-distance of 18S rDNA = 4.0%). The cells of Suigetsumonas keniensis are of similar size to those of Suigetsumonas clinomigrationis. Suigetsumonas keniensis bears a slightly shorter posterior flagellum than S. clinomigrationis. The cell itself has no trembling motion, but the anterior flagellum beats rapidly.

Haloplacidia gen. nov. Rybarski, Nitsche & Arndt

Description: Roundish to kidney shaped organisms, slightly elongated, uninucleate with two flagella, posterior flagellum smooth, anterior flagellum equipped with flagellar hairs (mastigonemes). Usually attached to the substrate via posterior flagellum, anterior flagellum mostly beating very fast, swimming cells move in anterior direction with the

anterior flagellum anterograde, anterior part of the cell is elongated, forms a feeding opening. Cells have a very fast trembling movement, phagotrophic feeding, occurs in hypersaline environment. Branches phylogenetically separate and distant from all other genera of placidids.

Etymology: *Halo* referring to the halophilic character, *placidia* referring to its phylogenetic position among placidids (name, feminine).

ZooBank registration: urn:lsid:zoobank.org:act:C26635CF-B56D-426D-A759-9D0423BFEA44.

Type species: *Haloplacidia cosmopolita* Rybarski, Nitsche & Arndt, this publication

Haloplacidia cosmopolita sp. nov. Rybarski, Nitsche & Arndt; Fig. 1 (AC-AF); type strain: Heterotrophic Flagellate Collection Cologne HFCC 1211.

Description: Roundish to kidney-shaped cell (Fig. 1AC), length of 3.4 – 7.0 µm (min. - max.; mean ± SD = 5.3 ± 1.0 µm, n = 20), width of 2.0 – 4.1 µm (3.0 ± 0.6 µm, n = 20). Two flagella, long anterior flagellum (Fig. 1AE), length of 4.6 – 7.1 µm (6.3 ± 0.7 µm, n = 16), a shorter posterior flagellum, length of 4.9 – 7.7 µm (6.1 ± 0.8 µm, n = 20) (Fig. 1AD). Both flagella emerge from a shallow groove at the ventral side of the cell (Fig. 1AD). Nucleus located close to this groove (Fig. 1AC), diameter of 0.7 – 1.0 µm (0.8 ± 0.1 µm, n = 20). Anterior part of the cell elongated forming a feeding opening. Cells frequently attached to the substrate by posterior flagellum, anterior mastigoneme-bearing flagellum (Fig. 3I) oriented in the opposite direction creating a feeding current for bacterial uptake. Swimming cells present, move in anterior direction with the anterior flagellum anterograde. Cell and the anterior flagellum with very fast trembling movement. Representatives of this species tolerate salinity from 4 to 200 PSU (Fig. 5). Cysts present when the salinity reached concentrations higher than 200 PSU or at conditions of starvation (Fig. 2C).

Etymology: *cosmopolita,* referring to its potential cosmopolitan distribution (adjective).

Type material: An electron microscopy preparation of HFCC 1211 has been deposited in the Biology Centre of the Museum of Natural History in Upper Austria, Linz, number EVAR 2021/19. This material constitutes the name-bearing type (an hapantotype) of the species.

ZooBank registration: urn:lsid:zoobank.org:act:5866AA94-78DA-4D14-B034-2D003F9F127E.

Type locality: Salar de Atacama (23°17′18.2″S, 68°10′35.8″W), a hypersaline lake in Northern Chile at an altitude of 2301 m at a salinity of 132 PSU.

Type sequence: All sequences are deposited in the GenBank database: 18S rDNA to 28S rDNA: MW591538. Further genes and their accession numbers can be found in the supplementary material (Table S3)

Remarks: The closest related isolated strain is MESS13, isolated from Salt Spring Island in Canada (p-distance of 18S rDNA = 0.1%; GU170212) and Suigetsumonas clinomigrationis is the closest related described species, isolated from Lake Suigetsu in Japan (p-distance of 18S rDNA = 9.0%). Haloplacidia cosmopolita is one of the smaller placidids. H. cosmopolita bears an anterior and posterior flagellum relatively similar in length (average length 6.3 and 6.1 µm, respectively). The phylogenetically distant Allegra dunaii is the only other placidid showing this character. Cells show fast trembling movement nearly as fast as that of Wobblia.

Discussion

The class Placididea (Moriya et al. 2002) comprises small heterotrophic stramenopiles which are generally recognisable by a trembling motion and being temporarily attached to the substrate by their posterior flagellum. Up to now only four species in four genera had been described, which are clearly separated into molecularly confirmed clades (Moriya et al. 2002; Park and Simpson 2010). Our present studies of various hypersaline, marine and brackish waters from Chile, Germany, Kenya, Canada, Japan, Atlantic Ocean, and the Caribbean Sea showed that placidids harbour an unexpected high species number, mainly from extreme habitats. Representatives of the Atlantic Ocean, Japan and the Caribbean Sea form a marine clade at the base of the phylogenetic tree, reflecting the marine origin of placidids. Analyses of salinity tolerances of the different species indicate that this ecological parameter might be an important driver for species separation (Fig. 5). Marine species tolerated a salinity range of 5 to 84 PSU, brackish water species tolerated also very low salinities (0–84 PSU), while representatives of the athalassic clades show the broadest range of tolerance (from 20 to 250 PSU). Up to now, there are two marine clades, *Placidia*/*Placilonga* n. gen. and *Wobblia*/*Pendulomonas*, two athalassic clades, *Allegra* n. gen. and *Haloplacidia* n. gen., and one brackish water clade, *Suigetsumonas*.

The lack of sequences of placidids such as *Placilonga atlantica* and *Placidia azorensis* in large surveys of oceanic plankton (e.g. de Vargas et al. 2015) could be explained by their preference for a benthic lifestyle. On the other hand, the lack of placidid sequences might be partially explained by their specific habitat requirements: *Suigetsumonas clinomigrationis* for example was isolated from a meromictic brackish water lake (Takahashi and Ichimura 1968; Matsuyama 1973; Kondo and Okamura 2017). *Haloplacidia cosmopolita* and *Allegra dunaii* were isolated from the Salar de Atacama in Chile where the inflow of groundwater into the hypersaline salar creates very variable salinity conditions that require a specific adaptation to a wide range of salinities. Occasionally, we observed that under culture conditions, an increase in salinity due to evaporation induces the formation of small (4–7 µm) cysts which may allow their survival as dormant stages at non-optimal conditions supporting their dispersal (e.g., Rogerson and Detwiler 1999).

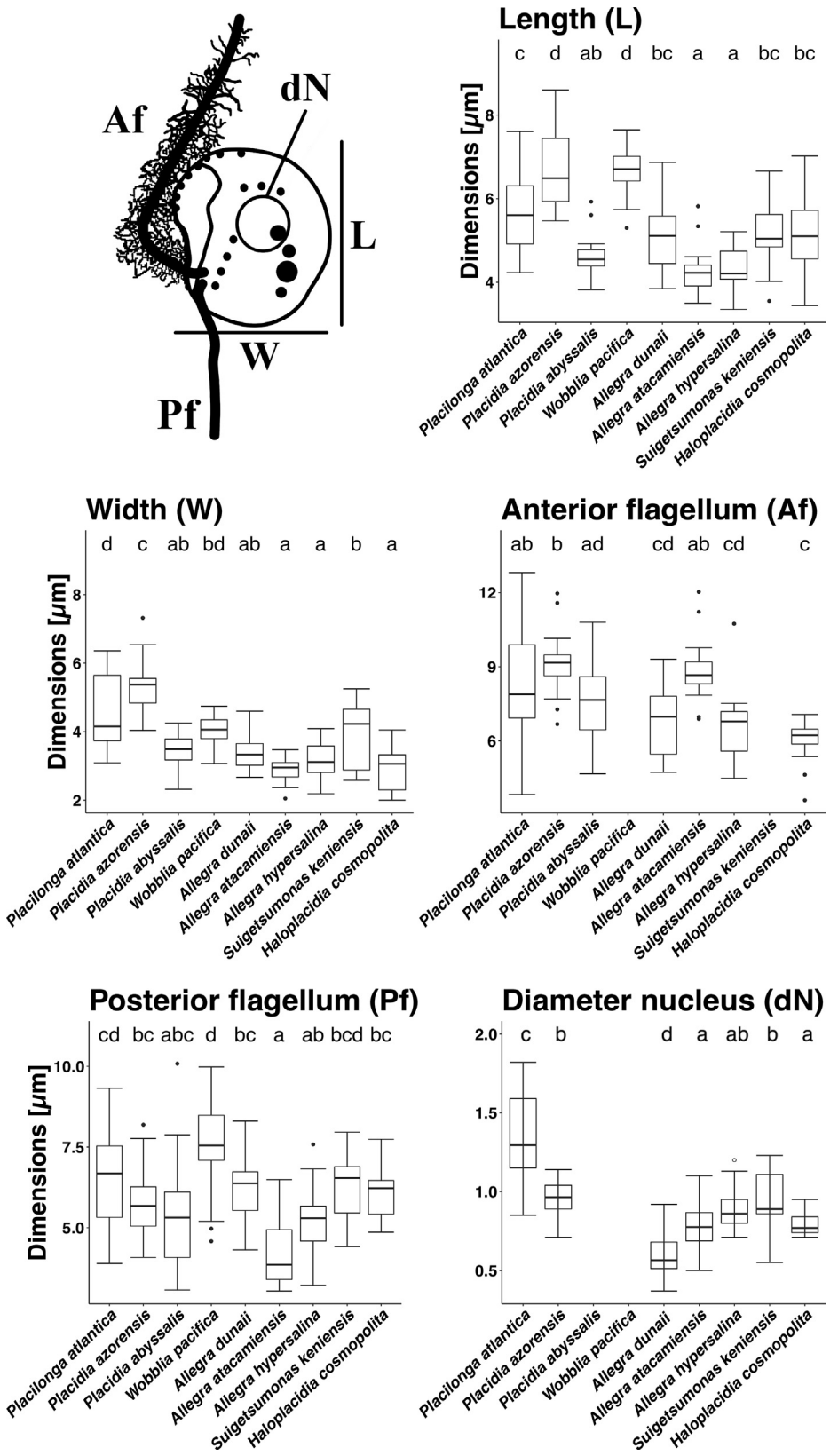

Fig. 6. Boxplots of morphological measurements of the newly described placidid species. Measurements of morphological features are indicated in the scheme in the upper left corner. Comparisons of measurements for the different species was carried out using ANOVA. Different letters indicate significant ($p < 0.05$) differences between measurements (Tukey´s post-hoc test; numbers of replicates are given in Table S1).

The analysis of transcriptome sequencing data of ten genes revealed a highly supported concatenated tree for Placidiidea broadly consistent in topology with trees obtained for of all individually analysed genes (Fig. 4, S2-S9). While the clustering into genera and species-level clades on the basis of molecular data is very robust, morphological differences among the different placidids species are rather small. Thus, we had to base our species differentiation mainly on the molecular identity of species. While most placidid species differed by more than 4% of the 18S rDNA base pairs (bp), there were three cases among the newly described species that differ by 2% of the 18S rDNA bp. A difference of 1% in 18S rDNA means already a difference of more than 15 bp, which is much more than the 1–2 bp error expected in Sanger sequencing (Shendure and Ji 2008). We followed the idea of Boenigk et al. (2012), stating that molecular data should be combined with morphological, ecological and behavioural characters of protists within a taxonomic framework. We compared the morphological character of placidids regarding the size (Fig. 6, Tab. S1), salinity tolerance as an ecological parameter (Fig. 5), and genetic difference based on the p-distance of the 18S rDNA (Tab. S2). Since the molecular difference between three species pairs is relatively low (1–1.5% p-distance) we here comment further on the validity of these species separations. 1) *Allegra atacamiensis/A. hypersalina*: The species differ in 18S rDNA by 1.5%, they are similar in cell size and salinity tolerance, but *A. hypersalina* has no mastigonemes on the anterior flagellum (Fig. 3G). In addition, the length of the posterior and anterior flagella differs slightly. *Allegra dunaii* differs to both *Allegra* species by a p-distance of more than 4% in the 18S rDNA 2) *Wobblia lunata/W. pacifica:* These differ by 1% in 18S rDNA, which means a difference of more than 15 bp, which is an order of magnitude larger than the sequencing error potentially introduced (Shendure and Ji 2008). The isolation of another population 330 km apart resulted in a genotype 100% identical to *W. pacifica* (Fig. 4), indicating the reliability of the usage of SSU rDNA for species delineation. In addition, *W. lunata* is larger than *W. pacifica*, and tolerates a higher range in salinity. 3) *Placidia cafeteriopsis/P. abyssalis*: These differ by 1.2% in 18S rDNA, *P. cafeteriopsis* is larger than *P. abyssalis* and tolerates a higher range of salinity. In the case of slight differences among genotypes that clustered together within one clade with described species, we refrain from describing new species at the present stage of knowledge, though isolates originate from very different habitats: *Haloplacidia cosmopolita* (13 strains, p-distance of 18S rDNA within this clade 0.1%), *Allegra dunaii* (7 strains, p-distance of 18S rDNA within this clade 0.1%), *Allegra atacamiensis* (2 strains, p-distance of 18S rDNA within this clade 0.1%) and *A. hypersalina* (3 strains, p-distance of 18S rDNA within this clade 0.1%). In these cases, further studies including the study of highly variable regions like the ITS should be performed in future. This could help to identify potentially cryptic species.

The separation of the newly established genus *Placilonga* from the genus *Placidia* was based on the high p-distance in the SSU rDNA (more than 12%), clearly separating these two taxa and indicating a long-term evolutionary separation. Though evolutionary rates vary among different protist groups (e.g., Parfrey et al. 2011), previous studies based on molecular clock analyses of 15 protein coding genes show, that this p-distance of 12% reflects an evolutionary separation of several million years (Arndt et al. 2020).

Due to the morphological similarities between the different species of placidids and the relatively high interspecific variation (Tab. S2), the sequence of *Pendulomonas adriperis* Tong, 1997 provided by Yubuki et al. (2015) for a strain isolated from Sooke, British Columbia (Canada), should be compared to isolates of its type location in Southampton Water (U.K). The present study of athalassic waters revealed a particular high number of different genotypes among placidids, offering an interesting study area for future evolutionary biological studies.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Authors contributions

Sampling in the Atacama Region was carried out by A.E. R., F.N., and H.A.; German and deep-sea strains were collected and isolated by H.A.; Canadian and Polish strains were isolated by J.S.P. and A.G.B.S.; P.F. isolated and analysed W. pacifica; S. clinomigrationis was isolated by R.K.;

molecular and morphological characterizations were carried out by A.E.R.; A.E.R. and F.N. performed the phylogenetic analyses. Comparative analyses of salinity tolerances were conducted by A.E.R.; A.E.R. and F.N. conducted bioinformatical analyses of transcriptomic data; project was launched and supervised by H.A.; A.E.R. and H.A. wrote the manuscript. All authors reviewed and revised the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejop.2021.125809.

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Supplementary Material

Revision of the phylogeny of Placididea (Stramenopiles):

Molecular and morphological diversity of novel placidid protists from extreme aquatic environments

Alexandra E Rybarski[1], Frank Nitsche[1], Jong Soo Park[2], Paulina Filz[1], Patricia Schmidt[1], Ryuji Kondo[3], Alastair GB Simpson[4], Hartmut Arndt[1]

Table legends

Table S1: Comparison of morphometric data of all new placidid species. For all features the mean ± SD is given; presence or absence of mastigonemes is indicated by +/-; AF – Anterior flagellum; PF – Posterior flagellum.

Taxon		Cell length (µm, n=20)	Cell width (µm, n=20)	AF length (µm, n=20)	PF length (µm, n=20)
Placilonga atlantica	HFCC 1505	5.7 ± 0.9	4.6 ± 1.1	8.3 ± 2.5	6.6 ± 1.6
Placidia azorensis	HFCC 952	6.7 ± 1.0	5.3 ± 0.8	9.1 ± 1.3	5.7 ± 1.1
Placidia abyssalis	HFCC 745	4.6 ± 0.5	3.5 ± 0.5	7.6 ± 1.7	5.4 ± 1.7
Wobblia pacifica	HFCC 834	6.7 ± 0.6	4.0 ± 0.5	na	7.6 ± 1.5
Allegra dunaii	HFCC 1261	5.1 ± 0.7	3.4 ± 0.5	6.7 ± 1.4	6.2 ± 1.0
Allegra atacamiensis	HFCC 5011	4.3 ± 0.6	2.9 ± 0.3	8.8 ± 1.2	4.2 ± 1.0
Allegra hypersalina	HFCC 1413	4.3 ± 0.6	3.1 ± 0.6	6.7 ± 1.3	5.3 ± 1.0
Suigetsumonas keniensis	HFCC 1412	5.2 ± 0.8	3.9 ± 1.0	na	6.3 ± 1.1
Haloplacidia cosmopolita	HFCC 1211	5.3 ± 1.0	3.0 ± 0.6	6.3 ± 0.7 (n=16)	6.1 ± 0.8

Taxon		Nucleus diameter (µm, n=20)	Cyst length (µm, n=20)	Cyst width (µm, n=20)	Mastigonemes
Placilonga atlantica	HFCC 1505	1.3 ± 0.3 (n=10)	na	na	+
Placidia azorensis	HFCC 952	1.0 ± 0.1	4.3 ± 1.1	4.7 ± 1.2	+
Placidia abyssalis	HFCC 745	na	na	na	+
Wobblia pacifica	HFCC 834	1.2 ± 0.1	na	na	+
Allegra dunaii	HFCC 1261	0.6 ± 0.1	na	na	+
Allegra atacamiensis	HFCC 5011	0.8 ± 0.1	na	na	+
Allegra hypersalina	HFCC 1413	0.9 ± 0.1	na	na	-
Suigetsumonas keniensis	HFCC 1412	1.0 ± 0.2 (n=17)	na	na	+
Haloplacidia cosmopolita	HFCC 1211	0.8 ± 0.1	na	na	+

Table S2: Calculations of SSU rDNA p-distances for all placidids.

	p-distance 18S rDNA	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44
1	***Placilonga atlantica* - HFCC 1505**																																												
2	***Placidia azorensis* - HFCC 952**	12.6																																											
3	*Placidia cafeteriopsis* - NIES-1013	12.5	2.0																																										
4	*Placidia cafeteriopsis* - NIES-1014	13.0	2.0	0																																									
5	*Placidia cafeteriopsis* - JF834161	12.8	2.4	1.7	1.7																																								
6	***Placidia abyssalis* - HFCC 745**	12.3	1.7	1.2	1.2	1.5																																							
7	*Placidia abyssalis* - HFCC 739	12.3	1.7	1.2	1.2	1.5	0																																						
8	*Wobblia lunata* - AB032606	15.8	14.8	15.2	15.0	15.1	15.4	15.4																																					
9	*Wobblia lunata* - NIES-1015	14.8	14.8	14.6	15.0	14.8	14.7	14.7	1.2																																				
10	***Wobblia pacifica* - HFCC 834**	14.9	15.1	14.7	15.2	15.0	14.8	14.8	2.2	1.0																																			
11	HFCC 5000	14.9	15.1	14.8	15.2	15.0	14.8	14.8	2.2	1.0	0																																		
12	*Pendulomonas adriperis* - KM816649	15.5	14.5	15.0	15.0	14.8	14.8	14.8	3.0	2.7	3.2	3.2																																	
13	KT861125	15.5	15.7	16.3	16.3	16.0	15.8	15.8	4.6	3.7	3.8	3.8	0.8																																
14	AY520454	15.1	14.9	14.7	15.2	15.0	14.6	14.6	3.7	2.7	3.1	3.2	0.9	0.3																															
15	***Allegra dunaii* - HFCC 1261**	17.1	14.4	14.2	14.7	14.7	14.1	14.1	11.8	10.8	11.0	11.2	11.3	13.4	10.9																														
16	HFCC 1252	17.0	14.3	14.2	14.7	14.6	14.1	14.1	11.9	10.9	10.9	11.2	11.4	13.7	11.0	0.2																													
17	HFCC 1258	17.1	14.4	14.2	14.7	14.7	14.1	14.1	11.8	10.8	11.0	11.2	11.3	13.4	10.9	0	0.2																												
18	GU170208 - ME8	16.9	14.3	14.2	14.6	14.6	14.1	14.1	11.8	10.8	10.8	11.1	11.3	13.6	10.9	0.2	0.1	0.2																												
19	HFCC 1242	17.8	14.4	14.7	14.7	14.8	14.5	14.5	11.6	11.6	11.7	11.7	11.4	13.4	11.6	0.1	0.2	0.1	0.2																										
20	HFCC 5009	17.0	14.3	14.2	14.6	14.6	14.1	14.1	11.8	11.0	11.2	11.2	11.3	13.6	11.1	0.1	0.1	0.1	0.1	0.1																									
21	HFCC 1221	17.1	14.3	14.2	14.7	14.7	14.2	14.2	11.8	10.8	10.9	11.2	11.4	13.6	10.9	0.1	0.1	0.1	0.2	0.2	0.1																								
22	***Allegra atacamiensis* - HFCC 5011**	17.3	14.8	14.8	15.2	15.2	14.7	14.7	12.3	11.4	11.5	11.5	11.6	12.7	11.5	4.7	4.7	4.7	4.7	5.0	4.6	4.6																						
23	HFCC 1400	17.9	14.8	15.2	15.2	15.3	15.1	15.1	12.1	11.8	11.9	11.9	11.6	12.9	11.7	4.9	4.9	4.9	4.9	5.1	4.9	4.9	0.1																					
24	HFCC 5015	17.5	15.0	15.1	15.1	15.2	15.0	15.0	11.7	11.4	11.4	11.4	11.3	12.7	11.4	4.2	4.4	4.2	4.4	4.4	4.3	4.3	1.5	1.5																				
25	HFCC 5021	17.1	15.0	14.7	15.1	15.1	14.6	14.6	11.9	11.0	11.1	11.1	11.3	12.7	11.2	4.1	4.2	4.1	4.2	4.4	4.2	4.2	1.5	1.5	0																			
26	***Allegra hypersalina* - HFCC 1413**	16.9	15.0	14.6	15.1	15.0	14.5	14.5	11.8	10.8	10.9	11.1	11.4	12.8	11.0	4.1	4.2	4.1	4.2	4.5	4.2	4.1	1.5	1.6	0.1	0.1																		
27	JX296580	14.8	14.9	14.8	15.1	14.9	14.8	14.8	10.5	9.6	9.8	9.9	9.9	10.0	9.8	10.6	10.8	10.6	10.7	11.2	10.7	10.6	10.8	11.2	11.1	10.8	10.6																	
28	*Suigetsumonas clinomigrationis* - NIES-3647	13.8	14.3	14.2	14.7	14.3	14.0	14.0	10.4	9.3	9.5	9.7	10.2	10.4	9.9	10.8	10.9	10.8	10.8	11.5	10.9	10.8	11.3	11.7	11.5	11.2	10.9	5.9															
29	*Suigetsumonas clinomigrationis* - AB976561	14.1	14.5	14.6	14.8	14.4	14.3	14.3	10.1	9.5	9.7	9.8	10.2	10.3	10.1	11.1	11.2	11.1	11.1	11.6	11.2	11.1	11.5	11.8	11.7	11.5	11.3	6.0	0.2													
30	***Suigetsumonas keniensis* - HFCC 1412**	14.6	14.7	14.6	15.2	14.6	14.5	14.5	10.7	9.7	9.8	10.0	10.4	10.3	9.9	10.7	10.8	10.7	10.8	11.4	10.8	10.6	11.0	11.4	11.4	11.0	10.8	5.7	3.8	4.0												
31	KX465214	14.7	14.7	14.8	15.2	14.8	14.6	14.6	10.7	9.8	9.9	10.1	10.5	10.1	10.0	11.0	11.1	11.0	11.1	11.7	11.2	10.9	11.2	11.5	11.6	11.3	11.0	5.7	4.1	4.2	0.5											
32	HFCC 207	14.8	13.6	13.8	14.1	14.0	13.5	13.5	9.5	8.3	8.5	8.6	9.3	10.9	9.2	9.8	9.8	9.8	9.7	10.4	9.9	9.8	10.2	10.5	10.5	10.2	10.0	8.3	8.8	9.0	8.8	8.9										
33	HFCC 1408	14.8	13.6	13.8	14.1	14.0	13.6	13.6	9.5	8.5	8.8	8.8	9.3	10.9	9.4	10.0	10.0	10.0	9.9	10.4	9.9	10.0	10.3	10.5	10.5	10.3	10.2	8.3	9.0	9.2	9.0	9.1	0									
34	GU170207 - ME5	14.8	13.6	13.8	14.1	14.0	13.5	13.5	9.5	8.3	8.5	8.6	9.3	10.9	9.2	9.8	9.8	9.8	9.7	10.4	9.9	9.8	10.2	10.5	10.5	10.2	10.0	8.3	8.8	9.0	8.8	8.9	0	0								
35	HFCC 1222	15.3	13.6	14.3	14.3	14.2	14.0	14.0	9.3	8.9	9.1	9.1	9.5	10.9	9.7	10.4	10.4	10.4	10.3	10.6	10.3	10.4	10.7	10.7	10.7	10.7	10.6	8.6	9.4	9.4	9.4	9.4	0.1	0.1	0.1							
36	HFCC 881	15.1	13.6	14.1	14.1	14.0	13.8	13.8	9.2	8.7	8.9	8.9	9.3	10.9	9.5	10.2	10.2	10.2	10.2	10.4	10.2	10.2	10.5	10.5	10.5	10.5	10.4	8.4	9.2	9.2	9.1	9.2	0	0	0	0.1						
37	HFCC 209	15.3	13.6	14.1	14.2	14.1	13.9	13.9	9.2	8.8	9.0	9.0	9.4	10.9	9.6	10.3	10.3	10.3	10.3	10.5	10.3	10.3	10.6	10.7	10.7	10.6	10.5	8.5	9.3	9.3	9.2	9.3	0	0	0	0.1	0					
38	GU170212 - MESS13	14.8	13.6	13.8	14.1	14.0	13.5	13.5	9.5	8.3	8.5	8.6	9.3	10.9	9.2	9.8	9.8	9.8	9.7	10.4	9.9	9.8	10.2	10.5	10.5	10.2	10.0	8.3	8.8	9.0	8.8	8.9	0	0	0	0.1	0	0				
39	***Haloplacidia cosmopolita* - HFCC 1211**	14.8	13.6	13.8	14.1	14.1	13.6	13.6	9.5	8.2	8.5	8.6	9.3	10.8	9.1	9.8	9.8	9.8	9.7	10.4	9.9	9.8	10.2	10.5	10.5	10.2	10.0	8.2	8.8	9.0	8.8	8.9	0.1	0.1	0.1	0.1	0.1	0.1	0.1			
40	HFCC 1407	14.8	13.6	13.8	14.1	14.0	13.5	13.5	9.5	8.3	8.5	8.6	9.3	10.9	9.2	9.8	9.8	9.8	9.7	10.4	9.9	9.8	10.2	10.5	10.5	10.2	10.0	8.3	8.8	9.0	8.8	8.9	0	0	0	0.1	0	0	0	0.1		
41	HFCC 1409	14.8	13.6	13.8	14.1	14.0	13.5	13.5	9.5	8.3	8.5	8.6	9.3	10.9	9.2	9.8	9.8	9.8	9.7	10.4	9.9	9.8	10.2	10.5	10.5	10.2	10.0	8.3	8.8	9.0	8.8	8.9	0	0	0	0.1	0	0	0	0.1	0	
42	HFCC 1247	14.7	13.8	14.7	14.7	15.1	14.4	14.4	9.5	8.7	8.7	8.7	9.3	10.9	9.9	11.4	11.2	11.4	11.1	11.4	11.2	11.3	11.5	11.6	11.4	11.4	11.3	8.7	9.2	9.2	9.0	8.8	0	0	0	0	0	0	0	0.1	0	0
43	HFCC 1410	17.9	16.2	16.8	16.8	16.7	16.6	16.6	10.5	9.8	9.8	9.8	10.3	14.0	10.7	11.3	11.2	11.3	11.1	11.5	11.2	11.3	11.3	11.4	11.5	11.5	11.4	9.1	9.8	9.8	10.2	10.4	0	0	0	0	0	0	0	0	0	0
44	GU170213 - MESS14	14.8	13.6	13.8	14.1	14.0	13.6	13.6	9.5	8.4	8.6	8.7	9.3	10.9	9.1	9.8	9.8	9.8	9.8	10.4	9.9	9.8	10.3	10.5	10.5	10.3	10.1	8.2	8.9	9.0	8.9	8.9	0.1	0.1	0.1	0.1	0	0	0.1	0.1	0.1	0.1	0

Table S3: Additional genes of placidids used for the calculation of the concatenated tree (α-tubulin, β-tubulin, EF1-α, Ef2, hsp90).

Species	Isolate	αtub	βtub	Ef1α	Ef2	hsp90
Placilonga atlantica	HFCC1505*	MZ440307	MZ440313	MZ440319	MZ440330	--
Placidia abyssalis	HFCC739*	MT042911	MT003213	MT042924	MT062933	MT048595
	HFCC745*	MZ440308	MZ440314	MZ440320	MZ440331	MZ440335
Wobblia pacifica	HFCC834*	MZ440309	MZ440315	MZ440321	MZ440332	MZ440336
Allegra dunaii	HFCC1221*	MT042913	MT003215	MZ440322	MT062935	MT048597
	HFCC1252*	MZ440310	MZ440316	--	--	MZ440337
	ME8*	MT042908	MT003211	MZ440323	MT062930	MT048592
	HFCC1258*	MT042914	MT003216	MT042927	MT062936	MT048598
	HFCC1261*	MT042915	MT003217	MZ440324	MT062937	MT048599
Allegra hypersalina	HFCC1413*	MT042920	MT003222	MT042931	MT062942	MT048604
Suigetsumonas keniensis	HFCC1412*	MZ440311	MZ440317	MZ440325	MZ440333	MZ440338
Haloplacidia cosmopolita	HFCC207*	MT042910	MT003212	MT042923	MT062932	MT048594
	ME5*	MT042907	MT003210	MT042921	MT062929	MT048591
	MESS13*	MT042909	MT178429	MT042922	MT062931	MT048593
	HFCC1211*	MT042912	MT003214	MZ440326	MT062934	MT048596
	HFCC1407*	MT042918	MT003220	MT042930	MT062940	MT048602
	HFCC1409*	MT042919	MT003221	MZ440327	MT062941	MT048603
	FURTHER PLACIDIDEA					
Wobblia lunata	NIES-1015*	MZ440312	MZ440318	MZ440328	MZ440334	MZ440339
Placidia cafeteriopsis	NIES-1013*	MT042916	MT003218	MZ440329	MT062938	MT048600
Suigetsumonas clinomigrationis	NIES-3647*	MT042917	MT003219	--	MT062939	MT048601

Figure legends

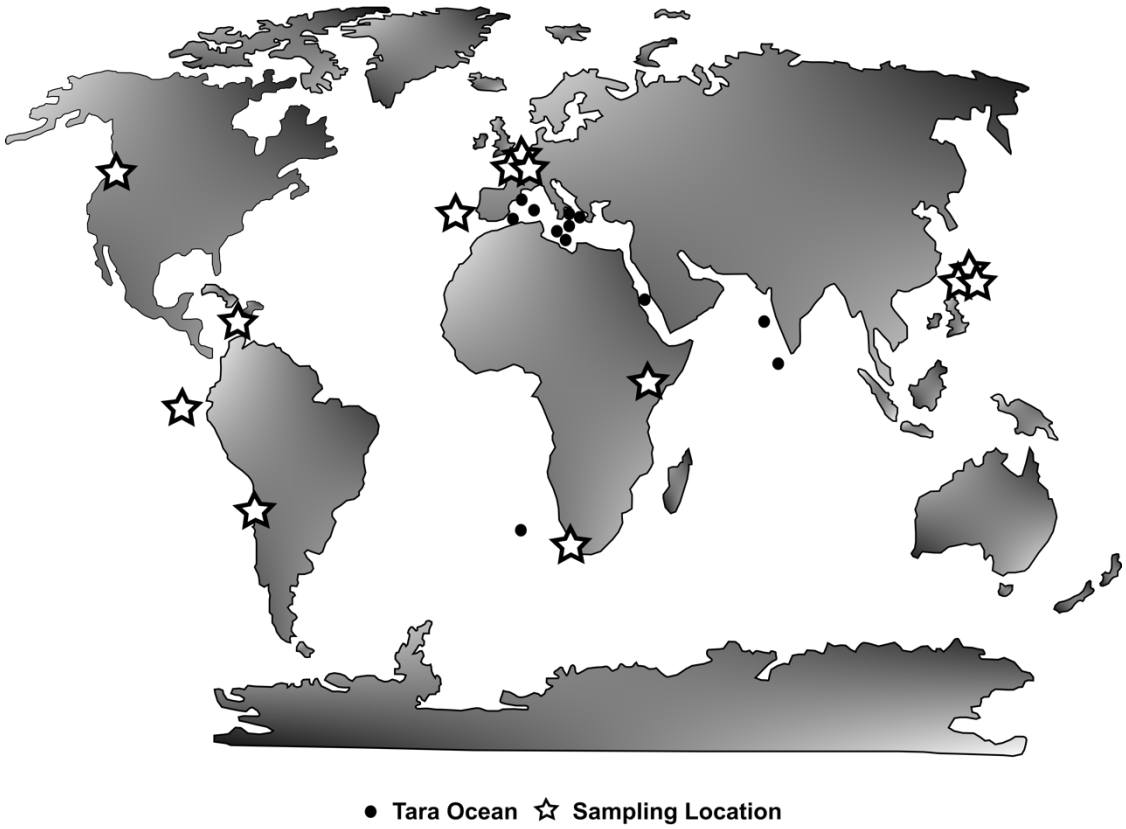
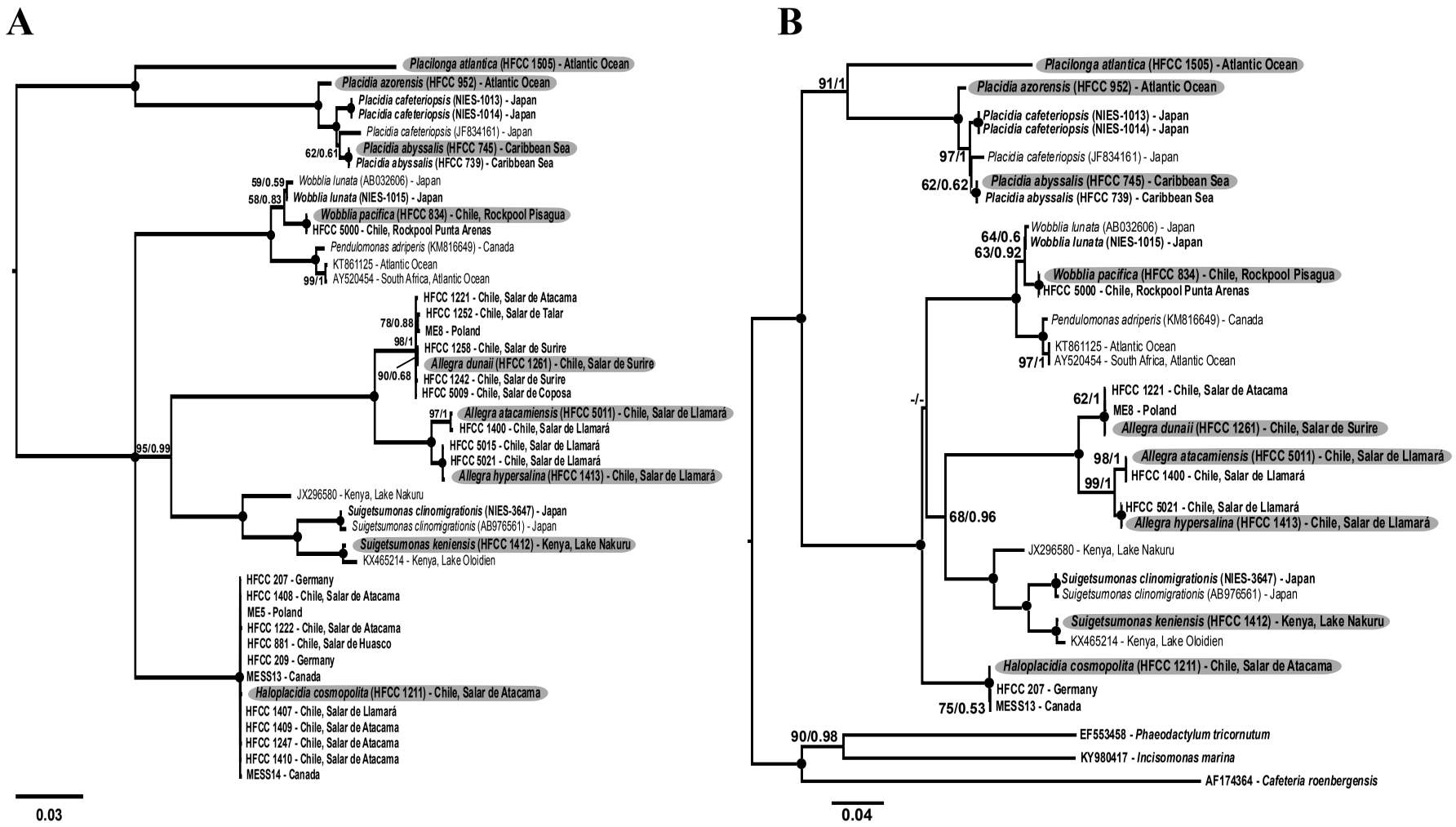


Figure S1: Global distribution of Placididea. Stars indicate sampling locations of this study. Black dots correspond to sampling positions of the Tara Ocean Expedition (de Vargas et al. 2015), where placidids were detected.

Figure S2: Phylogenetic trees of Placididea based on the SSU rDNA. ML bootstrap percentages (first value) and Bayesian posterior probabilities (PP: second value) are given for each node. Supporting values < 50 % or 0.5 are labelled with a dash (-). Solid circles indicate 100% bootstrap support and PP 1. A – rooted *Placilonga atlantica*, all isolated placidid strains are added to the calculation; B – rooted by three representatives as an outgroup; nearly identical sequences of placidid strains were removed for this analysis.



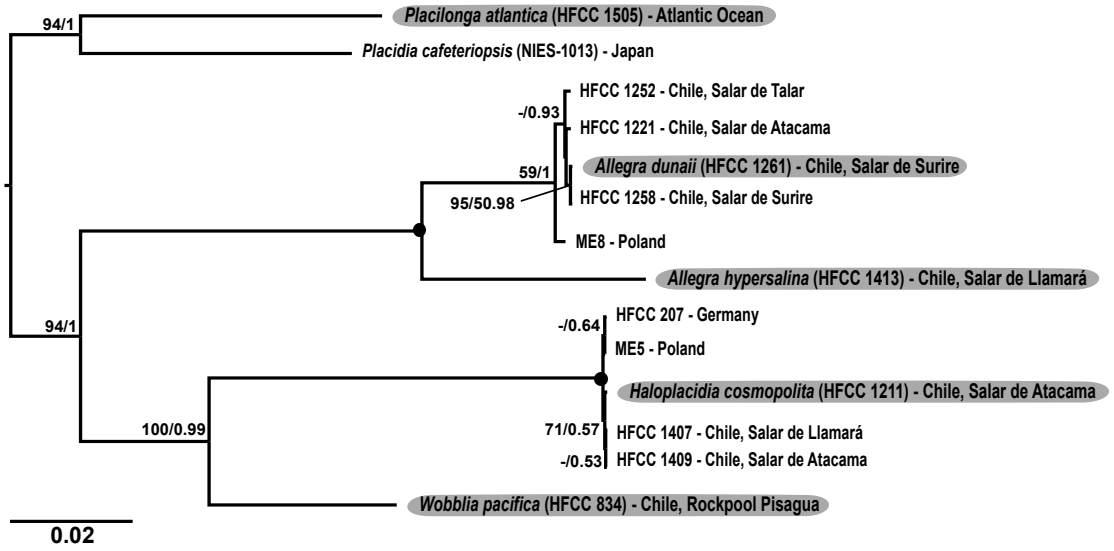


Figure S3: Phylogenetic tree of Placidiidea based on the ITS, 5.8S, ITS2. ML bootstrap percentages (first value) and Bayesian posterior probabilities (PP: second value) are given for each node. Supporting values < 50 % or 0.5 are labelled with a dash (-). Solid circles indicate 100% bootstrap support and PP 1.

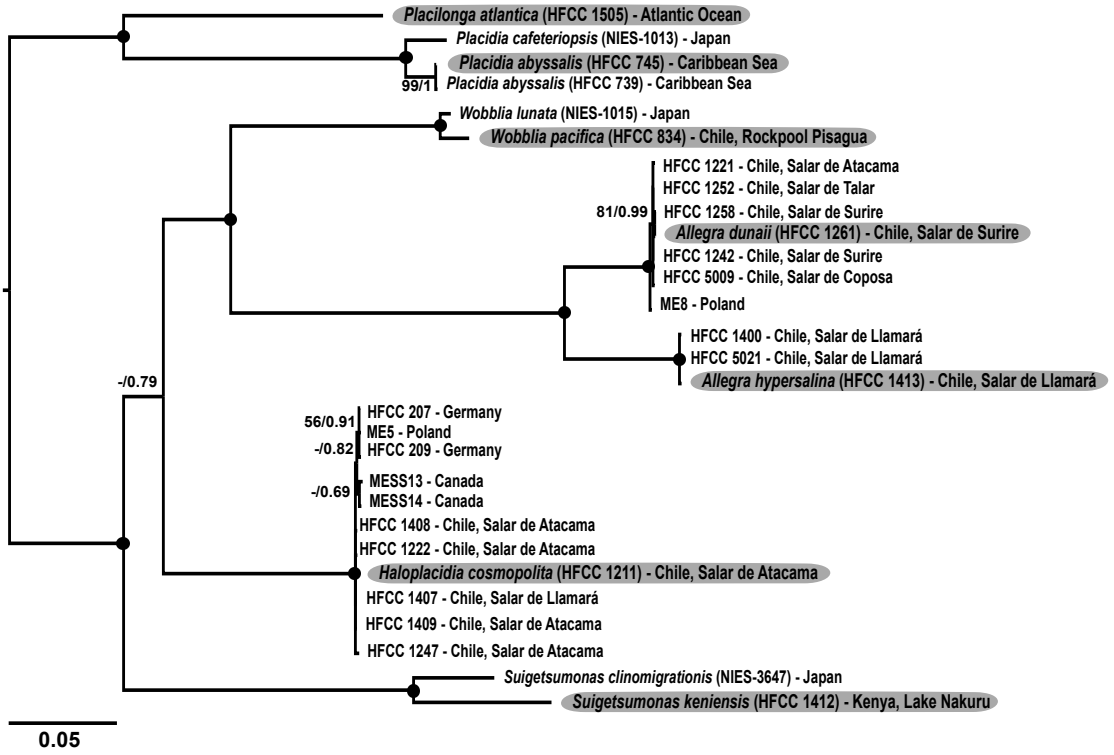


Figure S4: Phylogenetic tree of Placidiidea based on the LSU rDNA. ML bootstrap percentages (first value) and Bayesian posterior probabilities (PP: second value) are given for each node. Supporting values < 50 % or 0.5 are labelled with a dash (-). Solid circles indicate 100% bootstrap support and PP 1.

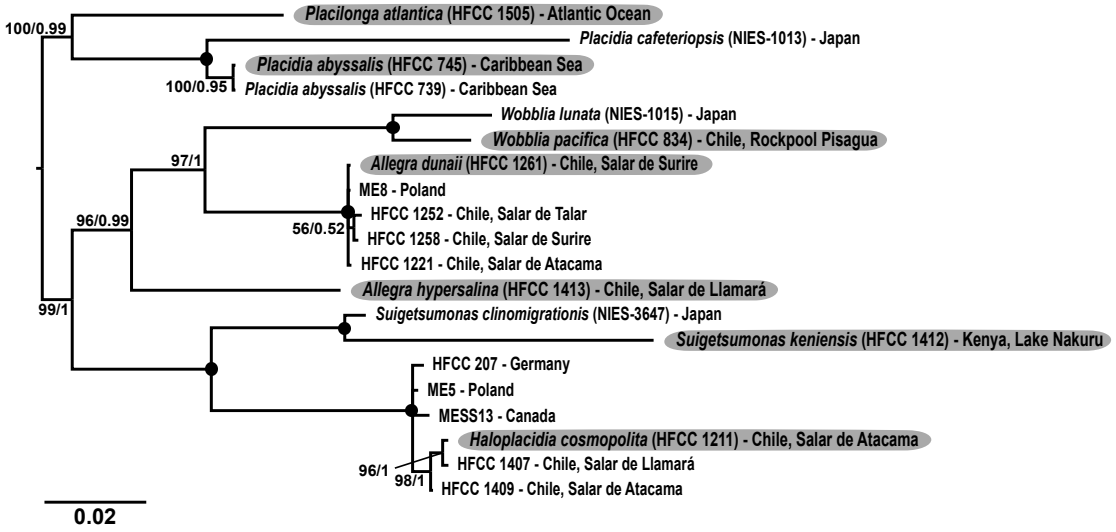


Figure S5: Phylogenetic tree of Placididea based on the alpha-1 tubulin. ML bootstrap percentages (first value) and Bayesian posterior probabilities (PP: second value) are given for each node. Supporting values < 50 % or 0.5 are labelled with a dash (-). Solid circles indicate 100% bootstrap support and PP 1.

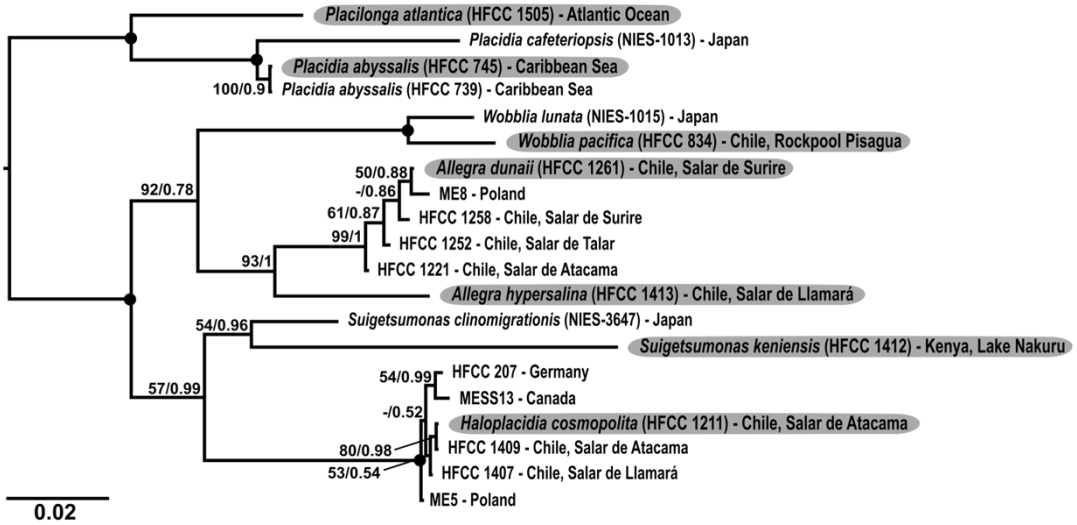


Figure S6: Phylogenetic tree of Placididea based on the beta-1 tubulin. ML bootstrap percentages (first value) and Bayesian posterior probabilities (PP: second value) are given for each node. Supporting values < 50 % or 0.5 are labelled with a dash (-). Solid circles indicate 100% bootstrap support and PP 1.

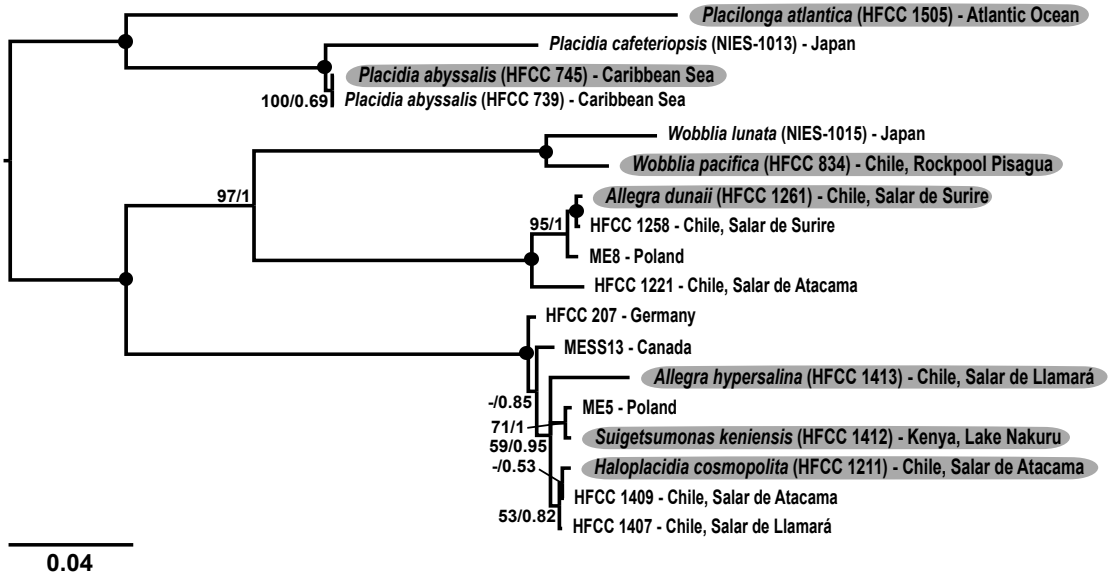


Figure S7: Phylogenetic tree of Placididea based on the translation elongation factor 1α (EF1-α). ML bootstrap percentages (first value) and Bayesian posterior probabilities (PP: second value) are given for each node. Supporting values < 50 % or 0.5 are labelled with a dash (-). Solid circles indicate 100% bootstrap support and PP 1.

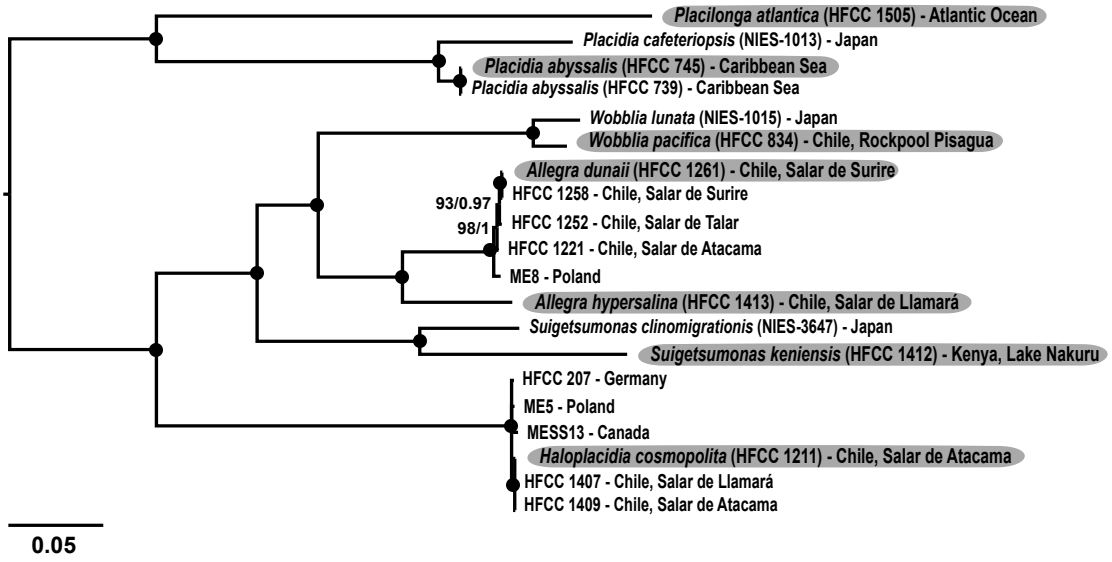


Figure S8: Phylogenetic tree of Placididea based on the translation elongation factor 2 (EF2). ML bootstrap percentages (first value) and Bayesian posterior probabilities (PP: second value) are given for each node. Supporting values < 50 % or 0.5 are labelled with a dash (-). Solid circles indicate 100% bootstrap support and PP 1.

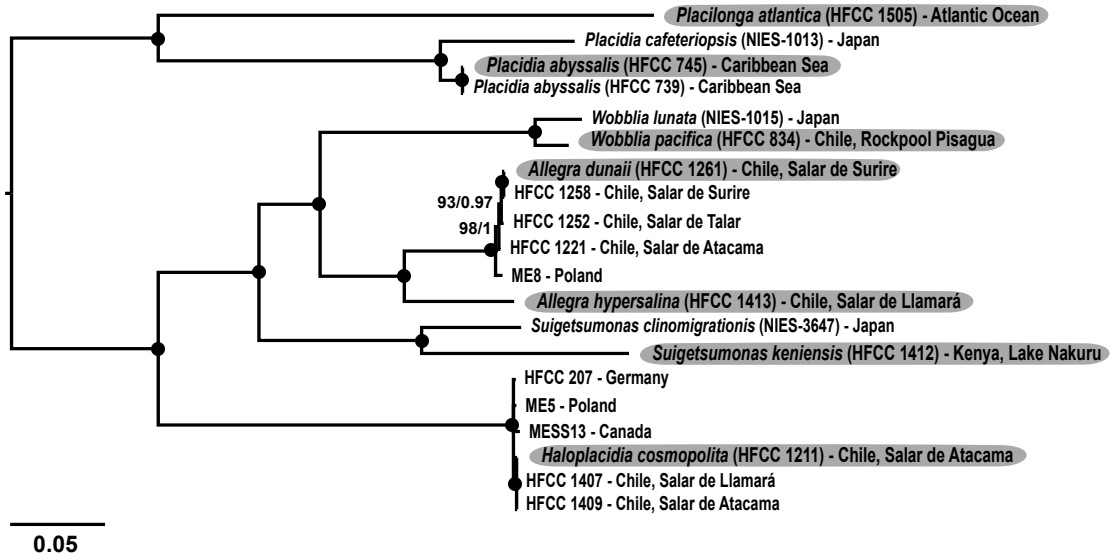


Figure S9: Phylogenetic tree of Placididea based on the heat shock protein 90 (hsp90). ML bootstrap percentages (first value) and Bayesian posterior probabilities (PP: second value) are given for each node. Supporting values < 50 % or 0.5 are labelled with a dash (-). Solid circles indicate 100% bootstrap support and PP 1.

Video legends

SV01 – *Placilonga atlantica* - HFCC_1505

SV02 – *Placidia azorensis* - HFCC_952

SV03 – *Placidia abyssalis* - HFCC_745

SV04 – *Wobblia pacifica* - HFCC_834

SV05 – *Allegra dunaii* - HFCC_1261

SV06 – *Allegra atacamiensis* - HFCC_5011

SV07 – *Allegra hypersalina* - HFCC_1413

SV08 – *Suigetsumonas keniensis* - HFCC_1412

SV09 – *Haloplacidia cosmopolita* - HFCC_1211

Part 1

Halophilic organisms from extreme environments

Chapter 2

Diversity and phylogeny of percolomonads based on newly discovered species from hypersaline and marine waters

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Diversity and phylogeny of percolomonads based on newly discovered species from hypersaline and marine waters

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Abstract

Percolomonads are common freshwater, marine and hypersaline tetraflagellated organisms. Current phylogenetic analyses of eukaryotes comprise only two species of this underinvestigated family. Here, we studied the morphology, salinity tolerance and 18S rDNA gene-based phylogeny of seven percolomonad cultures. We describe three new genera and five novel species of Percolomonadida based on phylogenetic distances and morphological characteristics: *Barbelia atlantica, B. abyssalis, Lula jakobsenorum, Nakurumonas serrata* and *Percolomonas doradorae*.

The new species show features typical for percolomonads, one long flagellum for skidding, three shorter flagella of equal length and a ventral feeding groove. The new species comprise organisms living in marine and athalassic hypersaline waters with salinity ranging from 10 to 150 PSU. Based on these novel taxa, the taxonomy and phylogeny of Percolatea was extended and further resolved.

Keywords: *Barbelia*; *Lula*; Molecular phylogeny; *Nakurumonas*; *Percolomonas*; Salinity tolerance

Introduction

Percolomonas is a common flagellate genus in marine and estuarine waters that is associated with sediment or lives in close association to suspended particles when occurring in the pelagial (e.g. Arndt et al. 2003). Cells have one long flagellum used for surface attachment and three shorter flagella for gathering particles into a feeding groove that takes up a large part of one side of the cell (Fenchel and Patterson 1986). The study of this taxon began when Fenchel and Patterson (1986) conducted a light and electron microscopy study of the already described organism *Tetramitus cosmopolitus* Ruinen 1938, which Ruinen (1938) had found to be present in numerous samples from Portugal, Australia, India and Indonesia. Fenchel and Patterson (1986) gave a detailed description of the species and placed this organism into the new genus *Percolomonas*. They considered it as a member of Heterolobosea incertae sedis. Later, Nikolaev et al. (2004) confirmed the assignment of *Percolomonas* to Heterolobosea based on molecular phylogenetics. Cavalier-Smith and Nikolaev (2008) and Yubuki and Leander (2008) demonstrated that *Percolomonas* is most closely related to the multiflagellated genus *Stephanopogon*. Here we follow the recent review of Heterolobosea systematics by Pánek et al. (2017), where *Percolomonas* was assigned to the family of Percolomonadidae and the genus *Stephanopogon* to the family Stephanopogonidae. The taxon Percolatea (originally proposed for

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Percolomonas alone) is sometimes used to encompass both taxonomic groups (Cavalier-Smith 2003).

Currently, six species have been described morphologically and assigned to the Percolomonadidae: *Percolomonas similis* Lee et al. 2003, *P. lacustris* Mylnikov 2016, *P. denhami* Tong 1997, *P. cosmopolitus* (Ruinen 1938) Fenchel and Patterson 1986, *P. spinosus* (Klug 1936) Larsen and Patterson 1990 and *P. sulcatus* (Stein 1878) Larsen and Patterson 1990. Originally, *Chilomastix cuspidata* (Larsen and Patterson 1990) Bernard et al. 1997 and *Carpediemonas membranifera* (Larsen and Patterson 1990) Ekebom et al. 1996 were assigned to the genus *Percolomonas*. Based on recent morphological and molecular studies (Bernard et al., 1997; Larsen and Patterson, 1990; Pánek et al., 2012) these species were transferred to newly separated genera together with *Harpagon decissus* (Perty 1852) Pánek et al. 2012 (originally assigned to *Tetramitus*, but later to *Percolomonas*). Among the up to now six described species, there are still uncertainties regarding their assignment to the genus *Percolomonas* (Pánek et al. 2017).

Considering the genotypes assigned to *Percolomonas* and a comparison of morphotypes being described as having a *Percolomonas*-like morphology (ventral groove, one long gliding flagellum, three shorter flagella of equal length) reveals a fundamental gap in our knowledge on this common and interesting genus. There is a large genetic distance (p-distance = 37.9%) between the two 18S rDNA gene sequences in GenBank assigned to *P. cosmopolitus* (AF011464 & AF519443). In former phylogenetic analyses, these sequences clustered in two long branches (Cavalier-Smith and Nikolaev, 2008; Pánek et al., 2017) clearly showing the need for an increased taxon sampling for this group.

We present here the description of five new percolomonad species and propose three new genera. Like Ruinen (1938) and Mylnikov (2016), we isolated also two strains from athalassic saline inland waters, although *Percolomonas* was previously mostly recorded from marine environments (e.g. Patterson and Lee 2000). Using the enlarged taxon sampling, we aimed to resolve evolutionary relationships within the distant *Percolomonas*-like taxa.

Material and methods

Isolation and cultivation

Seven *Percolomonas*-like strains were isolated from environmental samples, and monoclonal strains were established via the liquid-aliquot-method in artificial seawater medium (AB Reef Salt, Aqua Medic, Bissendorf, Germany) with corresponding salinities. Strain HFCC 219 was sampled from surface waters of the Azores taken with the yellow submarine LULA1000 (Rebikoff-Niggeler Foundation, Dive 049, in 2014); HFCC 2 and HFCC 3 were collected during a deep-sea expedition with *R/V* Meteor (M 48/1 in 2000); HFFC 1 was isolated from surface water samples from the Baltic Sea (Institute for Ecology, University of Greifswald, at Kloster, Hiddensee, in 1994); HFCC 1213 was collected from a brine shrimp pool at the Salar de Atacama, and HFCC 1236 from the Salar de Surire, both from Northern Chile taken March 2017, strain HFCC 1234 originated from water samples from the Nakuru Lake, Kenya, taken in September 2014 (Table 1). Strains were cultivated and maintained in 50 ml tissue culture flasks (Sarstedt, Germany) supplied with artificial seawater and one sterilized quinoa grain to stimulate bacterial growth.

Light microscopy

Morphological studies were carried out using an Allen Video-Enhanced Contrast (AVEC) system consisting of a Hamamatsu C6489 camera with noise suppression and contrast amplification (Argus-20, Hamamatsu, Japan) to record high-resolution stills of the strains. This was mounted on an inverted microscope (Zeiss Axio Observer A1) equipped with a 100×/1.4 NA oil immersion objective (DIC) and a water immersion condenser. Cultures were observed in Petri dishes with a coverslip base. Pictures were taken with VirtualDub-1.10.4 (www.virtualdub.org) and were edited by ImageJ (Abràmoff et al. 2004). Morphological characteristics (cell length, cell width, length of flagella; n = 25) of

Table 1. Sampling sites of the isolated percolomonads.

Species	Site	Coordinates	Salinity at sampling point (PSU)
***Percolomonas cosmopolitus* (HFCC 1)**	Hiddensee, Baltic Sea	54°35′0″N, 13°6′50″E	10
***Percolomonas doradorae* (HFCC 1213)**	Salar de Atacama, Chile	23°17′16″ S, 68°10′26″W	114
***Percolomonas* sp. (HFCC 1236)**	Salar de Surire, Chile	18°47′42″S, 69°05′07″W	50
***Nakurumonas serrata* (HFCC 1234)**	Lake Nakuru, Kenia	0°20′35″S, 36°5′32″E	5
***Lula jakobsenorum* (HFCC 219)**	North Atlantic, Azores, surface water	38°31′21″N, 28°40′6″W	36
***Barbelia atlantica* (HFCC 3)**	South East Atlantic, Angola Basin, surface water	19°6′0″S, 3°52′0″E	36
***Barbelia abyssalis* (HFCC 2)**	South East Atlantic, Angola Basin, deep sea (5392 m depth)	18°25′3″S, 4°44′0″E	36

specimens were measured with Axio Vision Rel. 4.8 (Zeiss, Germany).

Electron microscopy

For SEM preparation, 5 ml of each culture were fixed in 2.5% glutaraldehyde (prepared in Schmaltz-Pratt medium) and cacodylate buffer (final concentration of 0.05 M) for 30 min at 4 °C. After prefixation, 1% osmium tetroxide was added to the mixture for 10 min at room temperature. The samples were transferred on a filtration unit using polycarbonate filters (2 µm pore size) and dehydrated in an ethanol series with increasing concentrations of 30%, 50%, 70%, 80%, 90% and 96% by washing twice and remaining for 10 min in each alcohol concentration. After this step, a 50/50 hexamethyldisilazane (HMDS)–ethanol solution was added for 10 min followed by washing with 100% HMDS for 5 min as an alternative to critical point drying (Nitsche and Arndt 2008). The samples were air-dried on the filter, stuck to a sample holder and sputter-coated with a 120 Å layer of gold. The organisms were investigated morphologically at 15 kV using a scanning electron microscope (SEM; FEI Quanta 250 FEG).

DNA extraction and sequencing

DNA was extracted by using Quick gDNA Mini-Prep (Zymo Research Corporation, USA) and partial ribosomal 18S rDNA was amplified using the PCR primers 18SF50-Perc (5′ GTCCRAAGAYTAAGCCATGC 3′) and 18SRev-Perc (5′ CTTGATCCTTCTGCAGGTTCACC 3′). The initial denaturation step at 98 °C for 2 min was followed by 34 cycles at 98 °C for 2 min and two-step primer annealing at 55 °C for 30 s and additionally 52 °C for 45 s and strand elongation at 72 °C for 2:30 min followed by a final elongation for 10 min. PCR products were confirmed by agarose gel electrophoresis run, and DNA was purified using a PCR Purification Kit (Jena Bioscience, Germany). The sequences have been submitted to the GenBank database under accession numbers MZ264844-MZ264850.

Phylogenetic analyses

Phylogenetic analyses were conducted based on the 18S rDNA gene. The new percolomonad sequences were aligned with nine available sequences of *Percolomonas* in GenBank. Additionally, four *Stephanopogon* sequences and two outgroup sequences (from *Vahlkampfia* spp.) were added to the alignment. The alignment was carried out using ClustalW (Thompson et al. 1994) and manually corrected in BioEdit v7.2.5 (Hall 1999). After correction, the alignment encompassed 2200 unambiguously aligned nucleotides retained for phylogenetic analysis. The GTR +G- model for maximum likelihood analysis was selected according to MrAIC (Nylander 2004) and calculated with PhyML (Guindon and Gascuel 2003) using 1000 replicates for the bootstrap analysis. A Bayesian analysis was conducted using MrBayes, version 3.2.5. (Huelsenbeck and Ronquist 2001). Parameters of metropolis-coupled Markov Chain Monte Carlo (mcmc) runs were set to: ngen = 31000 (standard deviation of split frequencies dropped below 0.01 after 31000 generations), nchains = 4, samplefreq = 10, with other parameters at default settings. A conservative 25% burn-in was used. Pairwise distances of unmasked sequences were calculated using the pairwise alignment function within BioEdit after trimming sequences to the same length.

Salinity tolerance of percolomonad species

We performed experiments to determine the salinity tolerance in a range of 1–200 PSU of the different species. These experiments were similar to those of Park (2012); however, we used the occurrence of moving flagella of the organisms as an indicator, rather than sustained growth of the culture. Artificial salinity media was made by serial dilution of media #5 of Park (2012; stock solution: 137.6 g of NaCl, 3.8 g of KCl, 13.45 g of $MgCl_2 \cdot 6H_2O$, 1.65 g of $MgSO_4 \cdot 7H_2O$, 0.65 g of $CaCl_2 \cdot 2H_2O$) with deionised water to the appropriate salinity. 30 ml of stock solution were inoculated with 1 ml of exponentially growing cells and associated original bacteria with a sterilized wheat grain as a carbon source. All treatments were prepared in three replicates. For comparison with Park and Simpson (2015), we directly transferred stock cultures to the experimental medium without gradual salinity changes being aware that this procedure should be considered as a conservative estimate. The cultures were incubated at room temperature for eight weeks. Flasks were investigated every five days via light microscopy for actively moving cells as the indication of survival.

ZooBank registration

ZooBank registration number of present work: urn:lsid:zoobank.org:pub:53B9E1AF-A42D-4341-8AFD-08450BE8D064.

Results

General morphology of percolomonad flagellates

All seven strains possessed the typical percolomonad features, namely a ventral feeding groove and four flagella which insert at the head of the groove. One flagellum was long and attached to the substratum while the three shorter flagella were of equal length and beat in synchrony over the groove. The nucleus was mostly located in the anterior part of the cell body, while the posterior part was filled with food vacuoles (Ruinen 1938). For all studied species, cysts were observed in cultures. The seven strains represented six

species that differed in cell size and the length of flagella (Figs. 1–3; see also Taxonomic Summary); these were classified in four different genera (*Percolomonas*, and the new genera *Barbelia, Lula* and *Nakurumonas*) based on molecular data (see below).

Molecular phylogeny of percolomonad flagellates

We analyzed the partial 18S rDNA sequences of the seven monoclonal percolomonad cultures (shown in bold in the phylogenetic tree; Fig. 6). The phylogenetic analysis confirms the topology of previous studies based on Maximum likelihood (ML) as well as in the Bayesian analysis. The tree included 9 publicly available and 7 newly added percolomonad sequences. Sequences of *Percolomonas cosmopolitus* (AF519443), *P. cosmopolitus* (HFCC 1), *Percolomonas* sp. (HFCC 1236), *P. doradorae* (HFCC 1213), *P. lacustris* and several *Percolomonas* sp. strains formed a cluster with full bootstrap support, confirming their assignment to the genus *Percolomonas*. The sequence AF011464 from GenBank clusters together with *Barbelia abyssalis* and *B. atlantica* (strains HFCC 2, HFCC 3, respectively; both isolated from the Atlantic Ocean) with full bootstrap support, forming a basal cluster to all other Percolomonadida sequences. *Lula jakobsenorum* (HFCC 219) and *Nakurumonas serrata* (HFCC 1234) are positioned between the *Barbelia* and *Percolomonas* clade, with high bootstrap support.

Salinity tolerance

The species showed significant differences regarding their salinity tolerance (Fig. 7). The two marine *Barbelia* species had a narrow tolerance range; they only survived at salinities between 23 to 36 PSU. For the other marine percolomonad *Lula jakobsenorum,* survival was observed at salinities between 23 to 50 PSU. *Percolomonas* sp. survived at salinities between 23 to 60 PSU. The euryhaline *P. cosmopolitus* survived at brackish water and marine conditions. Movement could be observed at salinities between 3 PSU and 60 PSU and moving flagella were registered up to 75 PSU. *Nakurumonas serrata* showed the lowest salinity tolerance. It showed movements only at salinities between 3 to 30 PSU. The widest salinity tolerance range was

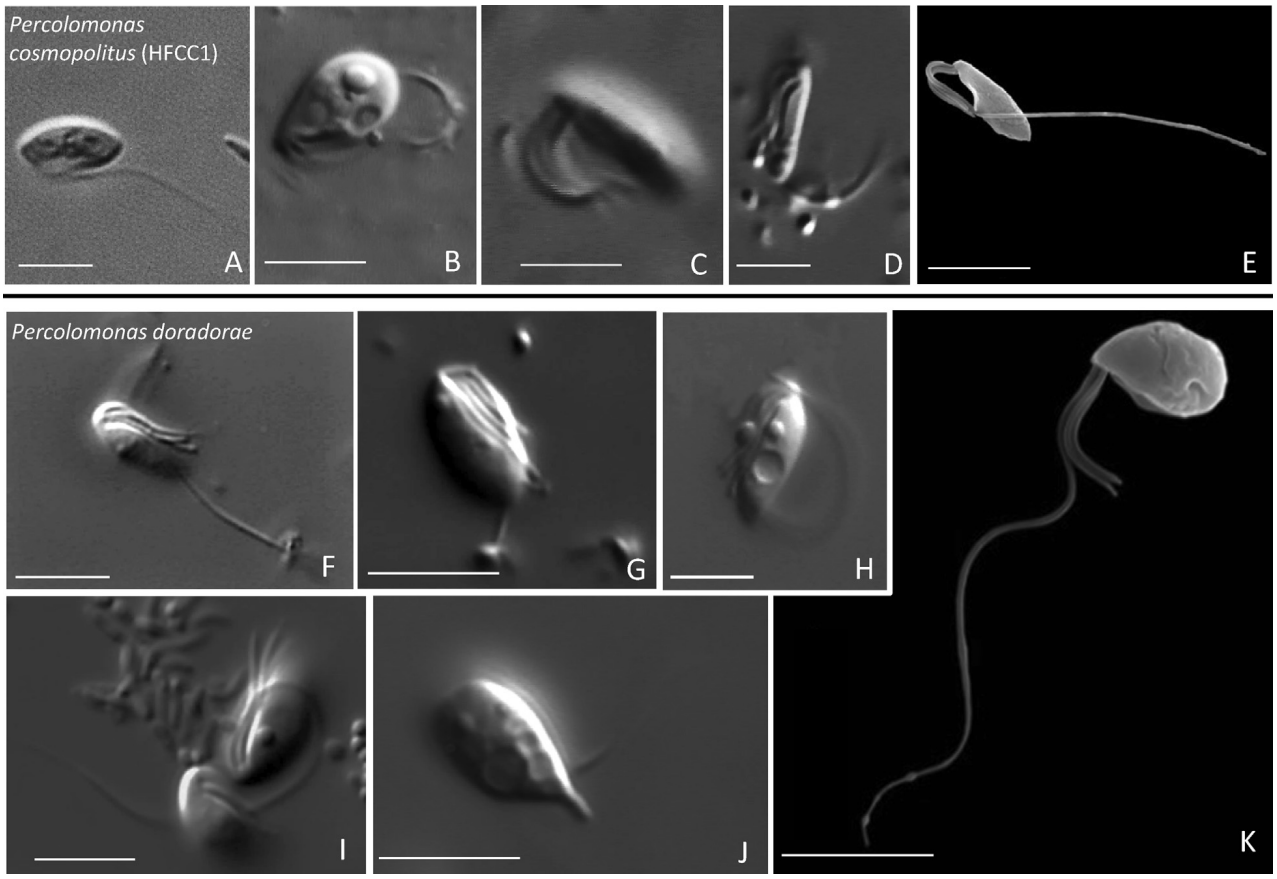

Fig. 1. Differential interference contrast (DIC) micrographs and scanning electron micrographs of *Percolomonas cosmopolitus* strain HFCC 1 and *P. doradorae.* (A-E) *Percolomonas cosmopolitus*: (A, B, D) DIC micrograph of whole cell, (C) short flagella, (E) EM micrograph of whole cell. (F-K) *P. doradorae*: (F, H, I) DIC micrographs of whole cells, (G) ventral feeding groove and short flagella, (J) cell shape, (K) EM micrograph of whole cell. Scale bar 5 μm.

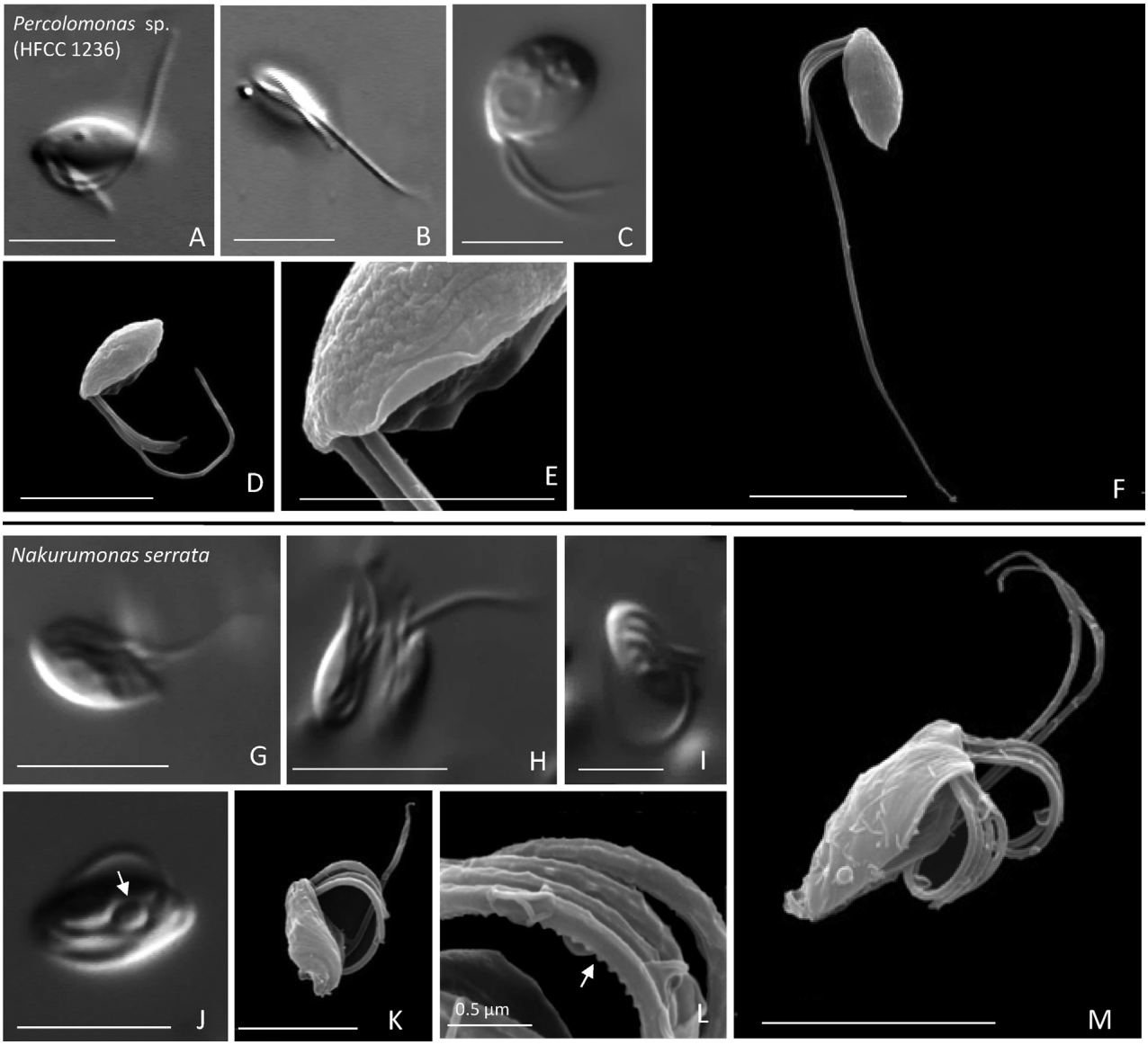

Fig. 2. Differential interference contrast (DIC) micrographs and scanning electron micrographs of *Percolomonas* sp. strain HFCC 1236 and *Nakurumonas serrata.* (A-F) *Percolomonas* sp. strain HFCC1236: (A-C) DIC micrographs of whole cell, (D-F) EM micrographs, (E) enlargement of D showing the ventral feeding groove and the velum. (G-M) *Nakurumonas serrata*: (G-J) DIC micrographs of whole cells, (H) dividing cell, (I) short flagella, (J) nucleus, (K-M) EM micrographs of whole cell, (L) row of teeth-like structure on short flagella, (M) EM micrograph of dividing cell. Scale bar 5 µm.

observed for *P. doradorae*. The strain could survive salinities ranging from 23 to 150 PSU (Fig. 7, Table 2).

Discussion

The position of *Percolomonas* within the heterolobosean was uncertain for a long time; Fenchel and Patterson (1986) proposed a position of *Percolomonas* as Heterolobosea incertae sedis. Cavalier-Smith (1993) considered *Percolomonas* together with other Heterolobosea among the Percolozoa. The recent review by Pánek et al. (2017) placed percolomonads together with Stephanopogonidae (collectively, Percolatea) within the taxon *Tetramitia*. By analysing 18S rDNA gene sequences, we unveiled several new clusters among percolomonads. Our studies on seven novel strains of percolomonad flagellates from marine and hypersaline environments show clearly that the new strains are members of the percolatean clade. Up to now, no amoeboid stage has been reported in either Percolomonadidae or Stephanopogonidae. The newly described species share several morphological characteristics with *P. cosmopolitus* (Ruinen 1938) Fenchel and Patterson 1986 but differ in several other features (Table 2). All species have a roundish to oval cell body shape with four flagella originating from the ventral surface anterior to a ventral feeding groove. In general, three flagella are of equal

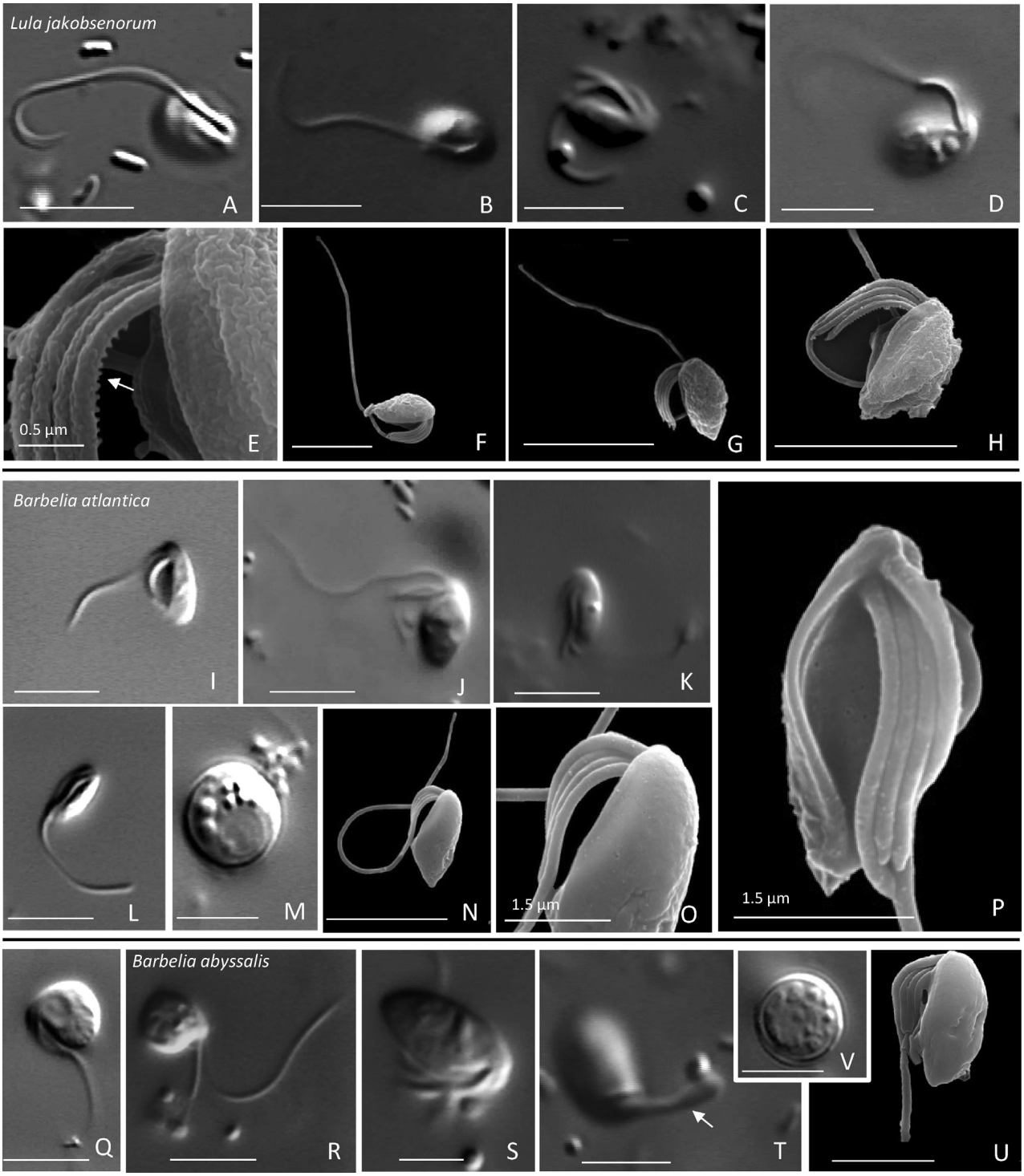

Fig. 3. Differential interference contrast (DIC) micrographs and scanning electron micrographs of *Lula jakobsenorum, Barbelia atlantica* and *B. abyssalis.* (A-H) *Lula jakobsenorum*: (A, B) DIC micrographs of whole cell, (C) short flagella, (D) cell shape, (E) enlargement of H showing row of teeth-like structure on short flagella, (F, G) EM micrograph of whole cell, (H) cell body with short flagella and ventral groove. (I-P) *Barbelia atlantica*: (I) ventral feeding groove, (J, K) DIC micrograph of whole cell, (L) long flagellum, (M) cyst, (N) EM micrograph of whole cell, (O) enlargement of N showing short flagella, (P) ventral feeding groove and short flagella. (Q-U) *Barbelia abyssalis*: (Q) DIC micrograph of whole cell, (R) long flagellum, (S) cell shape, (T) pseudopod, (U) electron micrograph of whole cell, (V) cyst. Scale bar 5 µm.

Table 2. List of percolomonads and their morphological characteristics.

Species	Reference	Flagella (number and morphology)	Cell length [µm]	Distinctive features	Habitat	Salinity at sampling site	Range of salinity tolerance
Percolomonada							
Percolomonas cosmopolitus (Ruinen 1938) Fenchel and Patterson 1986	Ruinen (1938)	4: three of equal length (1/3–1 cell length), one 2–4 times longer than cell	8–14	cells slightly pointed, nucleus in center of the cell, groove half as cell length	Portugal (Setubal), Australian waters, Indonesia (Java, Madura), India (Mumbai)	3–350 PSU	n.d.
P. cosmopolitus, strain observed by Fenchel & Patterson	Fenchel and Patterson (1986)	4: three of equal length (1/3–1/2 cell length, according to drawings), one 2–3 times longer than cell	6–12	nucleus in center of the cell, ventral groove extending along anterior two-thirds of cell	Aarhus Bay (Baltic Sea)	28 PSU	n.d.
P. cosmopolitus, strain HFCC 1	Carduck et al. (this study)	4: three of equal length, one 2.5 times longer than cell	5.0–9.0	groove half as cell length	Hiddensee Island (Baltic Sea)	10 PSU	3–75
Percolomonas lacustris Mylnikov 2016	Mylnikov (2016)	4: three of equal length, one longer than cell	6.6–9.9	groove extends three-fourths of cell, acroneme formed by one of the short flagella and by the long flagellum	hypersaline inland lake (Russia)	36 PSU	n.d.
Percolomonas sp. strain HFCC 1236	Carduck et al. (this study)	4: three of equal length, one longer than cell	5.8–8.3	groove almost as long as cell, velum like extended groove in LM and SEM studies	hypersaline inland lake (Atacama, Chile)	50 PSU	23–60
Percolomonas doradorae Carduck, Rybarski, Nitsche, & Arndt, n. sp. (HFCC 1213)	Carduck et al. (this study)	4: three of equal length, one longer than cell	4.5–8.3	groove extending more than half the cell, cell body with slightly pointed tip can be elongated to form pseudopodia like structures	hypersaline inland lake (Atacama, Chile)	114 PSU	23–150
Nakurumonas serrata Carduck, Nitsche & Arndt, n. sp. (HFCC 1234)	Carduck et al. (this study)	4: three of equal length, one longer than cell	3.8–6.0	three short flagella as long as, or slightly shorter than the cell	saline inland lake (Lake Nakuru, Kenia)	5 PSU	3–30
Lula jakobsenorum Arndt, Nitsche & Carduck n. sp. (HFCC 219)	Carduck et al. (this study)	4: three of equal length, one longer than cell	3.0–6.0	groove almost as long as cell, short flagella shorter than cell	marine (North Atlantic Ocean, surface water)	36 PSU	23–50
Barbelia atlantica Arndt, Carduck & Nitsche, n. sp. (HFCC 3)	Carduck et al. (this study)	4: three of equal length, one longer than cell	2.4–5.2	groove extends almost the length of cell	marine (South Atlantic, surface water)	36 PSU	23–36
Barbelia abyssalis Arndt, Carduck & Nitsche, n. sp. (HFCC 2)	Carduck et al. (this study)	4: three of equal length, one longer than cell	3.0–6.0	groove extends half or less as cell body	marine (Atlantic Ocean, deep sea)	36 PSU	23–36
Tetramitia incertae sedis							
Percolomonas similis Lee et al. 2003	Lee et al. (2003)	2: one short, one longer than cell	5.0–7.0	groove extending anterior half of the body	marine (Pacific Ocean, surface water)	n.d.	n.d.

(*continued on next page*)

Table 2 (*continued*)

Species	Reference	Flagella (number and morphology)	Cell length [µm]	Distinctive features	Habitat	Salinity at sampling site	Range of salinity tolerance
Percolomonas denhami Tong 1997	Tong (1997)	3: one short, one longer than cell, on 3 times longer than cell	6.5–7.0	groove about three-quarters of the cell	marine (Pacific Ocean, surface water)	n.d.	n.d.
Percolomonas spinosus (Klug 1936) Larsen and Patterson 1990	Larsen and Patterson (1990)	4: one longer than cell, three as long as groove	13.0–20.0	groove more than half of the cell	anoxic freshwater pond (Germany)	n.d.	n.d.
Percolomonas sulcatus (Stein 1878) Larsen and Patterson 1990	Larsen and Patterson (1990)	4: four of equal length	n.d.	Groove extending one-third to two-third of cell, two flagella longer than the others and often directed ventrally (Brugerolle and Simpson 2004)	Peatlands bogs (Bohemia)	n.d.	n.d.

length, and the fourth longer flagellum is used for gliding or skidding movements on the substrate. *Nakurumonas serrata* and *Lula jakobsenorum* may possess rows of little tooth-like extensions on the surface of the short flagella (only visible in EM). We hypothesize that this feature supports particle handling by the enlarged surface. The row of teeth has not been observed in other species. Additionally, *N. serrata* is covered with spiral ridges on the cell body. The significant genetic differences among all newly described percolomonads (Supplement data S1) and the formation of distinct clusters are the criteria for the establishment of new genera and families within the Percolomonadida.

The careful morphological investigation of the genus *Percolomonas* by Fenchel and Patterson (1986), which was based on an isolate of *P. cosmopolitus* from the Baltic Sea, agreed in all morphological features with the original description of the species by Ruinen (1938). The type locations are not well defined in Ruinen´s publication. Our sequence was nearly identical to the sequence published by Nikolaev et al. (2004) for *P. cosmopolitus* from the White Sea (Supplement data S1). Their isolate was morphologically similar to the strain investigated by Fenchel and Patterson (1986). Nikolaev et al. (2004) already stated that the other GenBank sequence for "*Percolomonas*" (AF011464) belongs to another genus. According to our studies (Supplement data S1), this sequence falls within the clade of barbeliids. Regarding the similar morphology of strain HFCC 1 compared to the original description by Ruinen (1938) and due to the high sequence identity shared between HFCC 1 and the isolate AF519443 of Nikolaev et al. (2004) we assigned the strain HFCC 1 to the species *P. cosmopolitus.*

Other species which were originally assigned to the genus *Percolomonas* must be considered for possible reassignment into other genera (for a summary see Table 2 and Fig. 4): *P. similis* Lee et al. 2003 possesses only two flagella and has never been found again for a more detailed study; *P. denhami* Tong 1997 has only three flagella which are all of unequal length; *P. spinosus* (Klug 1936) Larsen and Patterson 1990 ranging in size between 13 and 20 µm is three times larger than all other described percolomonad species and needs further confirmation. Based on their occurrence in anoxic wastewater habitats, we assume that *P. spinosus* and *P. sulcatus* (Stein 1878) Larsen and Patterson 1990 might belong to another clade. A reassignment was already carried out for *Carpediemonas membranifera* (Larsen and Patterson 1990) Ekebom et al. 1996, *Chilomastix cuspidata* (Larsen and Patterson 1990) Bernard et al. 1997 and *Harpagon decissus* (Perty 1852) Pánek et al. 2012, which formerly were assigned to the genus *Percolomonas.*

The investigated percolomonad species significantly differed concerning their salinity tolerance. Ruinen (1938) recorded growth from "3% to saturated" salinity for *P. cosmopolitus*, though this statement covers enrich-

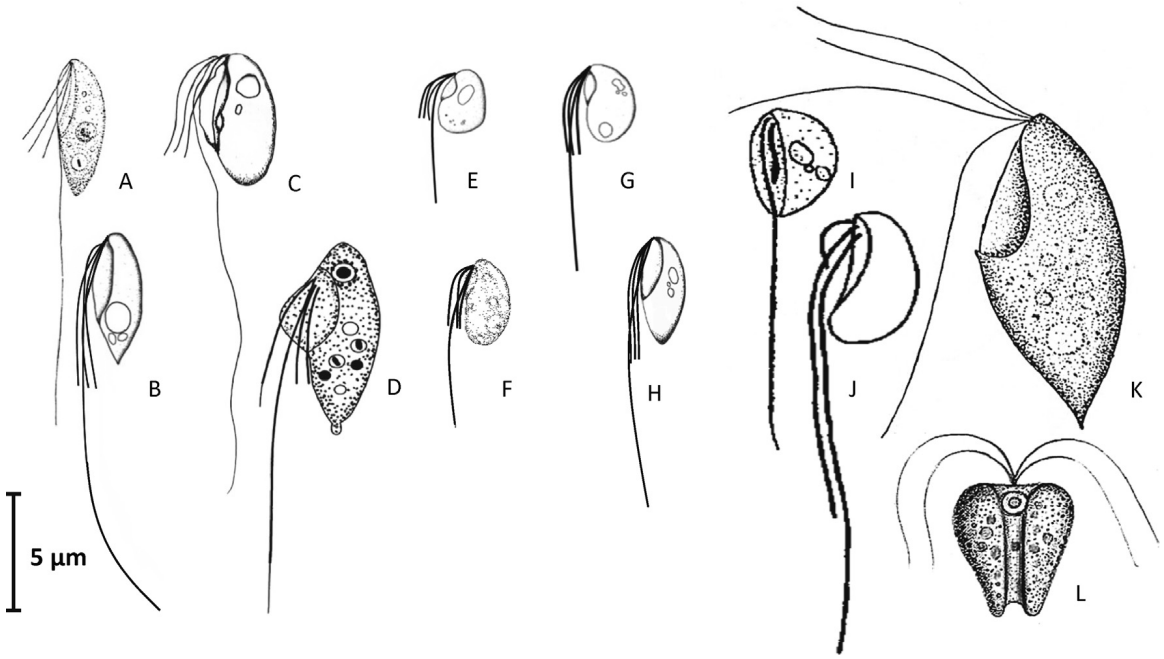

Fig. 4. Drawings of several percolatean flagellates. A: *Percolomonas cosmopolitus* (after Ruinen 1938); B: *P. doradorae* (orig.); C: *Percolomonas* sp. strain 1236 (orig.); D: *P. lacustris* (after Mylnikov 2016); E: *Barbelia abyssalis* (orig.); F: *B. atlantica* (orig.); G: *Lula jakobsenorum* (orig.); H: *Nakurumonas serrata* (orig.); I: *P. similis* (after (Lee et al., 2003)); J: *P. denhami* (after (Tong, 1997)); K: *P. spinosus* (after (Klug, 1936)); L: *P. sulcatus* (after (Stein, 1878)).

ments from many separate samples, not the growth range for a single isolate. Our strain of *P. cosmopolitus* (HFCC 1) grew at salinities of maximal 75 PSU. *Nakurumonas serrata* from Lake Nakuru in Kenya tolerates only relatively low salinities and seems to be phylogenetically closer related to the Chilean athalassic isolates (*Percolomonas* sp., *P. doradorae*). Even when living in inland waters, most of the strains did not lose their ability to survive at normal marine conditions. Heterolobosea includes a relatively large number of obligate halophiles (Park and Simpson, 2011; Park et al., 2007, 2009). There are at least three independent groups of halophiles, Tulamoebidae, Pharyngomonadidae and Percolomonadidae (Harding et al., 2016; Park and Simpson, 2011; Park et al., 2007, 2009). Our results confirm that Percolatea are not exclusively marine species, but also have representatives living in hypersaline inland water habitats as it was already found for *P. cosmopolitus* and *P. lacustris* (Ruinen, 1938; Mylnikov, 2016). Future detailed comparative molecular studies have to show whether *P. lacustris* isolated by Mylnikov (2016) from a site in southern Russia close to the border to Kazakhstan is similar or only closely related to our isolate from athalassic waters of the Atacama Desert in Chile. At least the salinity tolerance seems to be different between both strains. While the species *P. lacustris* (isolated by Mylnikov (2016) from athalassic waters) grows at salinities from 30 to 100 PSU (Tikhonenkov et al. 2019), our strain (isolated from the Atacama) showed a range of tolerance from 25-60 PSU in our experiments and did not survive at higher salinities (Fig. 7).

Percolomonads are phylogenetically much more diverse than previously thought. Future isolates will probably show that the *Percolomonas*-like morphology, characterized by a long gliding flagellum and the basket-like structure created by the three short flagella to transport bacteria towards the groove, was a successful evolutionary invention. This has led to the speciation of many taxa comprising many morphologically quite similar but genetically and probably also ecologically distant species.

Taxonomic summary

The ICZN (International Code of Zoological Nomenclature 1999) is used for the nomenclature.

Percolatea Cavalier-Smith 2003
Percolomonadida Cavalier-Smith 2003
Percolomonadidae Cavalier-Smith & Nikolaev 2008
Genus *Percolomonas* Fenchel & Patterson 1986
Species *Percolomonas doradorae* n. sp. Carduck, Rybarski, Nitsche, & Arndt; Figs. 1 (F-K), 4 (B); type strain: Heterotrophic Flagellate Collection Cologne HFCC 1213

Diagnosis: Free-living percolomonad, tetraflagellated 4–9 µm long and 2–5 µm wide, three short flagella of 3–6 µm in length and one longer flagellum of 8–19 µm in length; survival in salinities ranging from 23 to 150 PSU.

Etymology: *doradorae,* dedicated to the Chilean microbial ecologist Cristina Dorador for her pioneering work on salar microbial biology and her engaged help in the project (name, feminine).

Type locality: Hypersaline water of Salar de Atacama, Chile (23°17′16″ S, 68°10′26″W).

Type material: Electron microscopy preparation of HFCC 1213 has been deposited in the Biology Centre of the Museum of Natural History in Upper Austria, Linz, number EVAR 2021/7.

ZooBank registration: urn:lsid:zoobank.org:act:9BE8A0C6-BAF1-4D2A-BE93-9CA067EFFF16.

Description: *Percolomonas* species with a mean cell length of 6.2 ± 1.1 µm (4.6–8.3 µm, n = 19) and mean width of 3.3 ± 0.6 µm (2.3–4.4 µm; Fig. 5). The cell body has a slightly pointed tip at the end and can be elongated to form

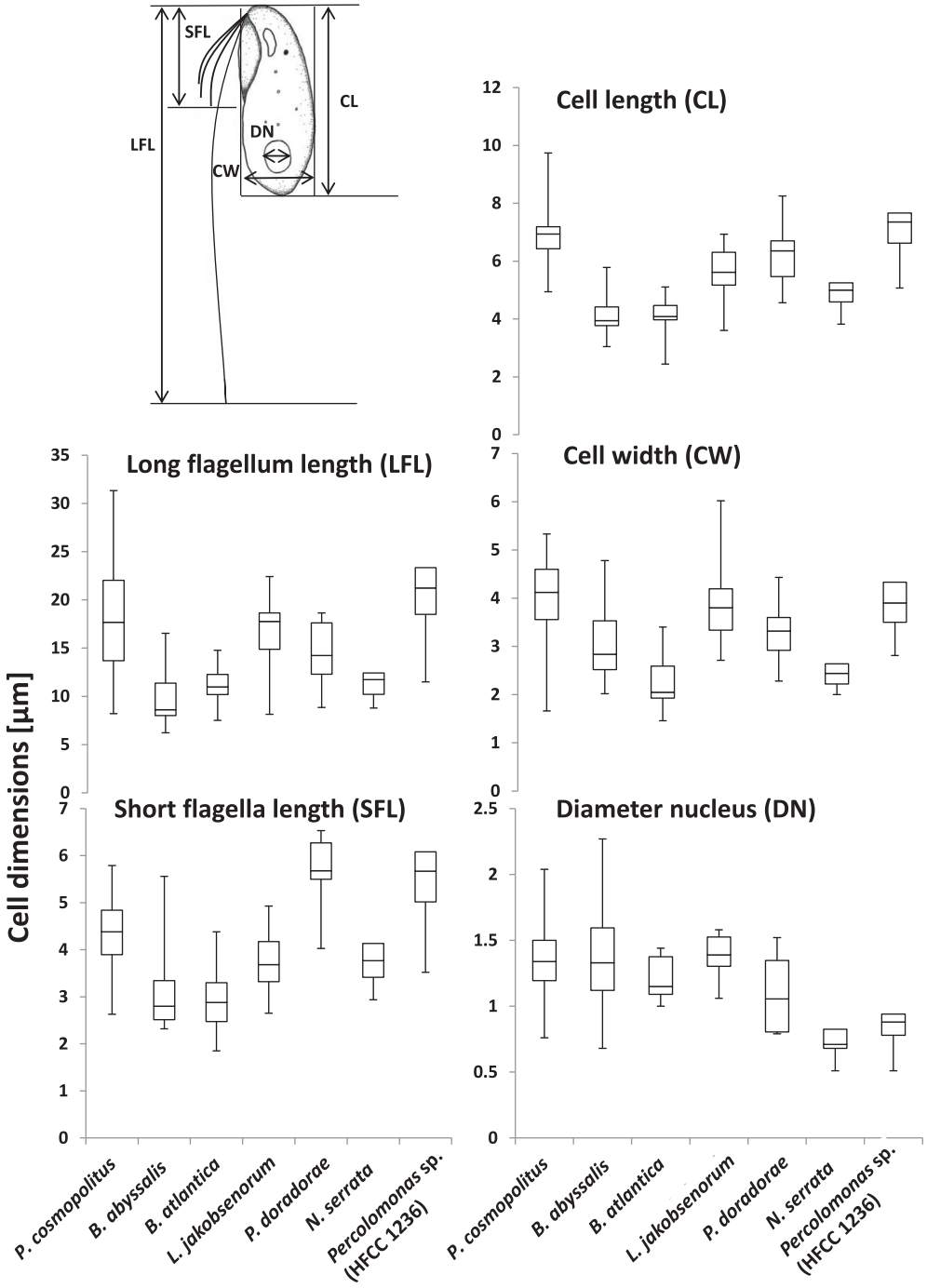

Fig. 5. Boxplots of morphological measurements of the new percolomonad strains. Measured characteristics were long flagellum length (LFL), Short flagella length (SFL) cell length (CL), cell width (CW) and the diameter of the nucleus (DN). Drawing shows how characteristics were measured.

pseudopodia like structures (Fig. 1 J). The long flagellum has a mean length of 14.9 ± 4.0 µm (8.9–18.6 µm), whereas the three flagella are as long as the cell body or slightly longer. The long flagellum is extended in a posterior direction and is used for skidding on the substratum. The three short flagella are commonly kept in an S-shape (Fig. 1 I). The prominent food vacuoles are well visible by light microscopy.

Remarks: Cell size of *P. doradorae* was found to be slightly smaller than described for the type species of the genus *P. cosmopolitus*, which had a cell length of 6–12 µm. Both species had elongated cell shapes with a pointed tip. The three short flagella were equally long as the cell body or slightly longer, whereas in contrast the short flagella were described to be as long as cell body or shorter for *P. cosmopolitus*. The feeding groove of *P. doradorae* extends to more than half of the cell as also found for *P. cosmopolitus* and *P. lacustris*. *Percolomonas doradorae* was found to be able to survive a very wide range of salinities in its environment (23–150 PSU). Closest 18S rDNA sequences in the phylogenetic analyses (Supplement data S1) were *Percolomonas* sp. strain LRS (MN105106) and *Percolomonas* sp. strain XLG1-P (MN105102) with p-distances of 0.12% and 1.2%, respectively. P-distances to the two previously described *Percolomonas* species *P. lacustris* and *P. cosmopolitus* were high with 18% and 21%, respectively.

Type sequence: The 18S rDNA gene sequence data of *P. doradorae* n. sp. has been deposited in the GenBank database with accession number MZ264844.

Genus *Nakurumonas* Carduck, Nitsche & Arndt, n. gen.

Description: Percolomonad flagellates from athalassic waters possessing three short flagella of equal length arising on the ventral part of the cell aiding in food particle collection, and a long flagellum. Short flagella are equally long or slightly shorter than the protoplast. The long flagellum is two to three times as longs as the cell. Cell shape is oval to elongated with a tip at the posterior end.

Type species: *Nakurumonas serrata* Carduck, Nitsche & Arndt, this publication

Etymology: *nakurumonas*, dedicated to the sampling site in Kenya (Lake Nakuru) and the people living in this region.

ZooBank registration: urn:lsid:zoobank.org:act:9359BBED-CE4A-4084-8456-3ED094F66900

Species *Nakurumonas serrata* Carduck, Nitsche & Arndt, n. sp.; Figs. 2 (G-M), 4 (H); type strain: Heterotrophic Flagellate Collection Cologne strain HFCC 1234

Diagnosis: Free-living percolomonad, tetraflagellated 3–6 µm long and 2–4 µm wide, three short flagella of 3–6 µm in length and longer flagellum of 8–15 µm in length. survival in salinities ranging from 3 to 30 PSU.

Etymology: *serrata,* due to the occasional presence of small teeth on the short flagella (adjective, feminine).

Type locality: Lake Nakuru, Kenya, (0°20′35″S, 36° 5′32″E).

Type material: Electron microscopy preparation of HFCC 1234 has been deposited in the Biology Centre of the Museum of Natural History in Upper Austria, Linz, number EVAR 2021/9.

ZooBank registration: urn:lsid:zoobank.org:act:A0C97EF4-E2DA-482D-A056-014C805FD07B

Description: Percolomonad with a mean cell length of 4.9 ± 0.5 µm (3.8–5.7 µm, n = 21) and mean width of 2.5 ± 0.3 µm (2.0–3.1 µm; Fig. 5). The long flagellum has a mean length of 11.3 ± 1.5 µm (8.8–14.4 µm), whereas the short flagella are 3.8 ± 0.5 µm in length (2.9–5.2 µm). The long flagellum is extended in a posterior direction and is used for skidding on the substratum. Cell body is oval to elongated. On the lower side of the short flagella small teeth-like structures were observed (Fig. 2L).

Remarks: With a mean cell length of 4.9 µm, *N. serrata* is smaller than representatives of the genus *Percolomonas*, like *P. cosmopolitus* with a cell length of up to 14 µm and *P. lacustris* with a cell length of 7–10 µm (Table 2). Small teeth-like structures on the three short flagella were observed (Fig. 2L). Similar structures were also observed for the species *Lula jakobsenorum* (Fig. 3E), but so far not in any species belonging to the genus *Percolomonas*. The three short flagella are equally long or slightly shorter than the protoplast. In contrast to other *Percolomonas* species, *N. serrata* was found to survive a relatively narrow range of salinities from 3 to 30 PSU (Fig. 7). As *P. doradorae*, *N. serrata* was isolated from athalassic water. Closest sequences in the phylogenetic analyses (Fig. 6) were *Lula jakobsenorum* (HFCC 219) and *Percolomonas* sp. strain P5-P (MN105103) with high p-distances of 33% and 34% (Supplement data S1).

Type sequence: The 18S rDNA gene sequence data of *Nakurumonas serrata* n. gen, n. sp. has been deposited in the GenBank database with accession number MZ264847.

Genus *Lula* Arndt, Nitsche & Carduck, n. gen.

Description: Marine percolomonad flagellates possessing three short flagella of equal length arising on the ventral part of the cell, which are as long as or slightly shorter than the protoplast and a long flagellum two to three times as long as the cell body. Ventral feeding groove extends to more than half of the cell length.

Type species: *Lula jakobsenorum* Arndt, Nitsche & Carduck, this publication

Etymology: *lula*, dedicated to the deep-sea studies of the submarine LULA1000 of the Rebikoff-Foundation around the Azores (name, feminine).

ZooBank registration: urn:lsid:zoobank.org:act:96311F29-B306-443C-AFA1-3F6843427B29

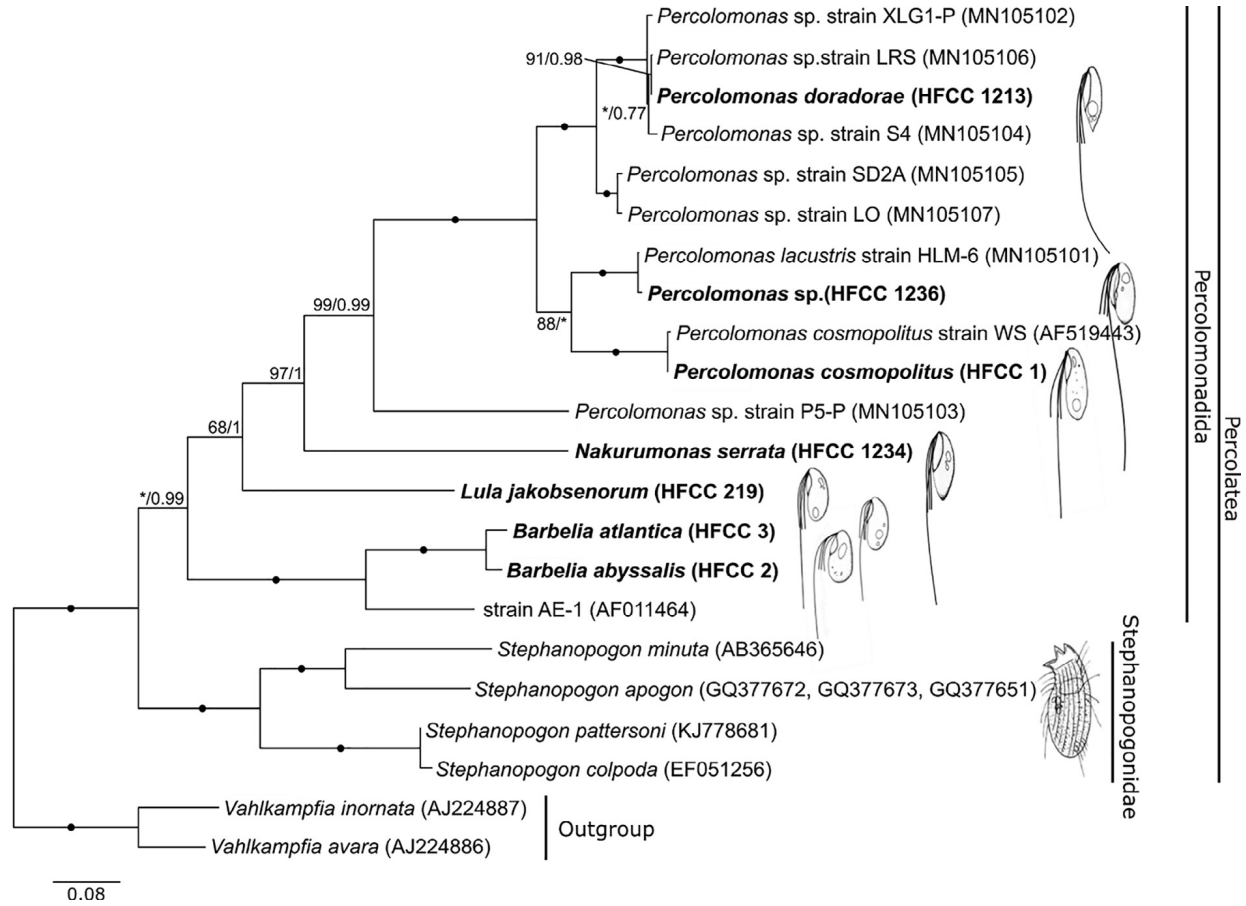

Fig. 6. Partial 18S rDNA gene tree (tree of Maximum likelihood analysis) of 2200 unambiguously aligned nucleotides (alignment manually corrected) showing the phylogenetic positions of *Percolomonas*-like morphotypes. Scale bar represents the expected substitutions per site in the ML analysis. The tree is outgroup-rooted with *Vahlkampfia*. Bootstrap values from Maximum Likelihood analysis (ML; 1,000 replicates) and Bayesian posterior probabilities (31,000 generations, with 25% burn-in) are shown at the nodes. Solid circles indicate 100% ML bootstrap support and Bayesian posterior probability of 1. *: Bootstrap value <50% and Bayesian posterior probability <0.7. Sequences with bold names mark the strains isolated in this study.

Species *Lula jakobsenorum* Arndt, Nitsche & Carduck, n. sp.; Figs. 3 (A-H), 4 (G); type strain: Heterotrophic Flagellate Collection Cologne strain HFCC 219.

Diagnosis: Marine water, free-living percolomonad, tetraflagellated with a protoplast 3–6 µm long and 2–5 µm wide, long flagellum of 6–17 µm in length and shorter posterior flagella of 2–5 µm in length; survival in salinities ranging from 23 to 50 PSU.

Etymology: *jakobsenorum,* dedicated to Kirsten and Joachim Jakobsen for their engagement in marine deep-sea research and their manifold support for the protistological studies (name, plural).

Type locality: Marine water of Atlantic Ocean, Azores, (38°31′21″N, 28°40′6″W)

Type material: Electron microscopy preparation of HFCC 219 has been deposited in the Biology Centre of the Museum of Natural History in Upper Austria, Linz, number EVAR 2021/8.

ZooBank registration: urn:lsid:zoobank.org:act:2C110805-13C2-4C1D-9E91-813F62EF5D0A

Description: Percolomonad species with a roundish cell shape with a mean cell length of 5.6 ± 0.9 µm (3.6–6.9 µm, n = 28) and mean width of 3.9 ± 0.7 µm (2.7–6.0 µm; Fig. 5). The long flagellum has a mean length of 16.3 ± 3.3 µm (8.1–22.4 µm), whereas the short flagella are 3.7 ± 0.6 µm (2.7–4.9 µm) long. The long flagellum is extended in a posterior direction and is used for skidding on the substratum. The short flagella are commonly kept in an S-shape (Fig. 3 D). Organisms make nutant movements of the cell body during skidding. On the lower side of the short flagella small teeth-like structures were observed (Fig. 3 E).

Remarks: With a mean cell length of 5.6 µm, *L. jakobsenorum* is slightly larger than *N. serrata* but still smaller than representatives of the genus *Percolomonas* (Table 2). Small teeth-like structures on the three short flagella were observed (Fig. 3 E). Similar structures were also observed for the species *N. serrata* (Fig. 2 L), but so far not in any species belonging to the genus *Percolomonas*. The ventral feeding groove of *L. jakobsenorum* extends to more than half of the cell length. Although, *L. jakobsenorum* was isolated from marine waters, the species was able to tolerate

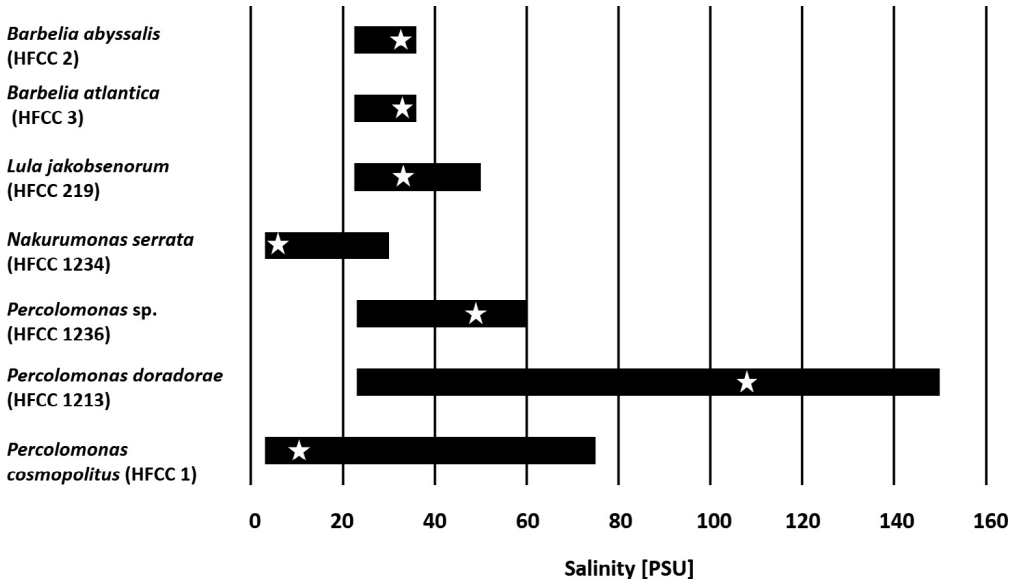

Fig. 7. Salinity tolerance of seven percolomonad strains. Response to various salinities (1–160 PSU). Salinities of the sites of isolation are marked with asterisks.

marine up to hyposaline conditions (Fig. 7). *L. jakobsenorum* was able to survive higher salinities (up to 50 PSU), than *N. serrata*, but was not as tolerant as representatives of the genus *Percolomonas*. Closest sequences in the phylogenetic analyses (Fig. 6) were *N. serrata* (HFCC 1234) and *Percolomonas* sp. strain P5-P (MN105103) with a high p-distance of 33% (Supplement data S1).

Type sequence: The 18S rDNA sequence data of *Lula jakobsenorum* n. sp. has been deposited in the Gen-Bank database with accession number MZ264848.

Genus *Barbelia* Arndt n. gen.

Description: Small marine percolomonad flagellates possessing three short flagella of equal length arising on the ventral part of the cell used for food particle collection, and a long flagellum used for gliding movements. Cells are roundish without a tip at the posterior end. Short flagella are as long as or slightly shorter than the cell. The long flagellum is more than two times the length of the cell.

Type species: *Barbelia atlantica* Arndt, Carduck & Nitsche, this publication

Etymology: *barbelia*, dedicated to Bärbel Arndt in appreciation of her invaluable support and patience in protozoological research and far beyond (name; feminine).

ZooBank registration: urn:lsid:zoobank.org:act:EF42D3A2-F911-4550-99AA-7965C35D1E6E

Species *Barbelia atlantica* Arndt, Carduck & Nitsche, n. sp.; Figs. 3 (I-P), 4 F; type strain: Heterotrophic Flagellate Collection Cologne HFCC 3.

Diagnosis: Marine, free-living percolomonad, tetraflagellated, 2–5 µm long and 2–4 µm wide, long flagellum of 7–15 µm in length and shorter posterior flagella of 2–5 µm in length. Survival at salinities ranging from 23 to 36 PSU.

Etymology: *atlantica,* dedicated to the sampling site in the Atlantic Ocean in the Angola trench (adjective).

Type locality: Surface water of the Atlantic Ocean, Angola basin (19°6′0″S, 3°52′0″E).

Type material: Electron microscopy preparation of HFCC 3 has been deposited in the Biology Centre of the Museum of Natural History in Upper Austria, Linz, number EVAR 2021/6.

ZooBank registration: urn:lsid:zoobank.org:act:33105240-99B9-4513-9D11-4DE8E0DEEE92

Description: Percolomonad species with a mean cell length of 4.1 ± 0.6 µm (2.4–5.1 µm, n = 21) and mean width of 2.2 ± 0.5 µm (1.5–3.4 µm; Fig. 5). The long flagellum has a mean length of 11.2 ± 1.7 µm (7.5–14.8 µm), whereas the three short flagella are 2.9 ± 0.9 µm in length (1.9–4.4 µm). The cell body is rounded and has no tip at the posterior end. The protoplast can be elongated, forming pseudopodia-like structures. The long flagellum is used for skidding on the substrate. The groove extends almost to the length of the protoplast (Fig. 3 P). The three short flagella undulate simultaneously in waves, reaching nearly the posterior end of the protoplast. *Barbelia atlantica* has a jerky movement, frequently shifting direction.

Remarks: With a mean cell length of 4.1 µm**,** *B. atlantica* is smaller than species belonging to the genera *Percolomonas* and *Lula* (Fig. 5). Cells of *B. atlantica* are roundish and have no tip at the posterior end. In contrast, a tip at the posterior end of the cell was described for *N. serrata* and species belonging to the genus of *Percolomonas* (Fig. 4). The ventral feeding groove of *B. atlantica* extends to more than half of the cell or is even found to be as long as the cell, as described already for other species of the Per-

colomonadida (*P. cosmopolitus, P. lacustris, P. doradorae, Percolomonas* sp. strain HFCC 1236). *Barbelia atlantica* was able to survive only a narrow range of salinity concentrations (23–36 PSU; Fig. 7), which corresponds to marine conditions. Closest sequences in the phylogenetic analyses (Fig. 6) were *B. abyssalis* (HFCC 2) and strain AE-1 (AF011464) with p-distances of 4% and 22%, respectively (Supplement data S1).

Type sequence: The 18S rDNA gene sequence of *Barbelia atlantica* n. sp. has been deposited in the GenBank database with accession number MZ264849.

Species *Barbelia abyssalis* Arndt, Carduck & Nitsche, n. sp.; Figs. 3 (Q-V), 4 (E); type strain: Heterotrophic Flagellate Collection Cologne strain HFCC 2.

Diagnosis: Marine free-living percolomonad, tetraflagellated, 3–6 µm long and 2–5 µm wide, three short flagella of 2–6 µm in length and longer flagellum of 6–17 µm in length; survival at salinities ranging from 23 to 36 PSU.

Etymology: *abyssalis,* according to the sampling site in the abyssal of the Atlantic Ocean in the Angola Basin (latin; feminine).

Type locality: Isolated from multicorer samples from Atlantic Ocean (Angola Basin; 18°25′3″S, 4°44′0″E) from a water depth of 5392 m.

Type material: Electron microscopy preparation of HFCC 2 has been deposited in the Biology Centre of the Museum of Natural History in Upper Austria, Linz, number EVAR 2021/5.

ZooBank registration: urn:lsid:zoobank.org:act:2E408535-83F1-4DB5-B982-9DA9118B2460

Description: Percolomonad species with a mean cell length of 4.1 ± 0.6 µm (3.1–5.8 µm, n = 21) and mean width of 3.0 ± 0.8 µm (2.0–4.8 µm; Fig. 5). The long flagellum has a mean length of 9.7 ± 2.6 µm (6.2–16.5 µm), whereas the three short flagella are 3.1 ± 1.0 µm in length (2.3–5.6 µm). The long, gliding flagellum is directed to the posterior end during movement on the substratum. Cell body without pointed posterior end. Pseudopodia formation present (Fig. 3 T). Cells show short distant shivery movements.

Remarks: Cell length of *B. abyssalis* did not differ significantly from the closely related species *B. abyssalis* (Fig. 5). However, the length of the three short flagella extended only to two-third of the protoplast and the ventral feeding groove of *B. abyssalis* is only half as long as the cell or even shorter. In contrast, in *B. atlantica* the ventral feeding groove extends almost over the whole cell. As *B. atlantica*, *B. abyssalis* was able to survive a narrow range of salinity concentrations with a range from 23 to 36 PSU corresponding to marine conditions. Closest sequences in the phylogenetic analyses were *B. atlantica* (HFCC 3) and strain AE-1 (AF011464) with p-distances of 4% and 22%, respectively (Supplement data S1).

Type sequence: The 18S rDNA gene sequence of *B. abyssalis* n. sp. has been deposited in the GenBank database with accession number MZ264850.

Author contributions

All authors were involved in the sampling, cultivation and isolation of the protists. S.C. conducted most of the experiments and the molecular and morphological characterizations. A.R. carried out some of the salinity tolerance studies. S.C., F.N., M.H. performed the phylogenetic analyses and the SEM studies. S.C. and H.A. wrote the manuscript, H.A. supervised the studies. All authors reviewed and revised the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejop.2021.125808.

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Part 1

Halophilic organisms from extreme environments

Chapter 3

Cafeteria in extreme environments: description and ecological investigations on *C. burkhardae* and three new species (*C. baltica*, *C. atacamiensis*, *C. paulosalfera*) from the Atacama Desert and the deep ocean

Cafeteria in extreme environments: description and ecological investigations on *C. burkhardae* and three new species (*C. baltica, C. atacamiensis, C. paulosalfera*) from the Atacama Desert and the deep ocean

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Abstract

The heterotrophic nanoflagellate genus *Cafeteria* has been found to be ubiquitously distributed in the marine realm. We could isolate and cultivate ten strains morphologically similar to *Cafeteria* from two extreme environments, the deep sea and meso to hypersaline waters of the Atacama Desert. Molecular analyses (18S rDNA, 28S rDNA) of newly isolated strains from the marine realm resulted in four more *Cafeteria burkhardae* strains from the deep North Atlantic Ocean and one new species (*C. baltica*) isolated from brackish waters of the Baltic Sea. The three strains isolated from the Atacama Desert belong to two new species (*C. atacamiensis* and *C. paulosalfera*). Morphological characterizations of these strains obtained by high resolution microscopy revealed only small differences to already described species. However, molecular analyses showed a clear separation of the different *Cafeteria* species. We exposed several strains to increasing salt concentrations (2-150 PSU) to investigate their salinity tolerance. Only the marine strains of *C. burkhardae* were able to survive at salinities up to 150 PSU, indicating the possibility to inhabit a broader spectrum of habitats including hypersaline environments besides the deep sea with its high hydrostatic pressure.

***Keywords**:* Autecology; Baltic Sea; Bicosoecida; North Atlantic Ocean; Phylogeny; rDNA; Salinity tolerance

Introduction

Heterotrophic nanoflagellates play a crucial role within the microbial food web as links to higher trophic levels and remineralizers of nutrients in biogeochemical cycles (Arndt et al., 2000; Azam and Malfatti, 2007; Landry and Calbet, 2004). While recent estimates indicate that protists encompass a range of 70,000-150,000 species, only a tiny fraction is yet known, and there is a high number of so far undescribed species (de Vargas et al., 2015; Grossmann et al., 2016; Mahé et al., 2017; Schoenle et al., 2021). One issue of cultivation-based methods is the requirement of specific cultivation conditions for most species and, as a result, we miss a majority of taxa during this approach (del Campo et al., 2013), especially when sampled from environments with extreme conditions such as the deep sea or salars in the Atacama Desert. Thus, high-throughput-sequencing (HTS) studies have become a common approach to investigate protist diversity from samples worldwide. Such HTS surveys recover novel eukaryotic lineages that have not been reported from cultures so far (del Campo et al., 2013; Edgcomb et al., 2011; López-García et al., 2001; Schoenle et al., 2021; Triadó-Margarit and Casamayor, 2013), making it challenging to get ecological and morphological information on the species inhabiting these environments. Thus, it is important to use a combination of cultivation-independent and cultivation-depended methods together with autecological experiments to describe the role of protists in their surroundings (Schoenle et al., 2016).

The bicosoecid species *Cafeteria burkhardae*, a bacterivorous heterotrophic nanoflagellate, has been repeatedly reported from the marine environment occurring in surface waters and the deep sea by cultivation techniques and high-throughput sequencing (Atkins et al., 2000; de Vargas et al., 2015; Larsen and Patterson, 1990; Massana et al., 2020; Patterson et al., 1993; Schoenle et al., 2020; Vørs, 1993). *Cafeteria* often occurs in cultivation studies together with other r-strategist such as *Caecitellus* and *Rhynchomonas* (Boenigk et al., 2002; Patterson

and Lee, 2000). It even has been cultivated from deep-sea sediment collected at 8,380 m depths despite the lack of high hydrostatic pressures during cultivation (Schoenle et al., 2020). *Cafeteria burkhardae* seems to have a cosmopolitan distribution occurring not only in cultures from all over the world's oceans, but it is also highly abundant in environmental metagenomic studies targeting the epi-, meso-, and bathypelagial (de Vargas et al., 2015; Massana et al., 2020) and in deep-sea sediments (Schoenle et al., 2020). Besides *C. burkhardae*, six new *Cafeteria* species have recently been described by morphological and molecular features from marine surface waters and the deep sea, including the Atlantic Ocean, Pacific Ocean, Mediterranean Sea, Indian Ocean and Baltic Sea (Schoenle et al., 2020). Pressure experiments with several *Cafeteria* strains isolated from surface waters and the deep sea showed that several *Cafeteria* strains are able to survive up to 500 bar, which is the prevailing pressure at 5,000 m depth, indicating their potential to thrive even under high hydrostatic pressure (Živaljić et al., 2018).

Another extreme environment for aquatic protists are deserts. We carried out investigations in the Atacama Desert, the oldest and driest non-polar terrestrial desert on Earth with aridic and hyperaridic conditions since the Late Jurassic 150 million years ago (Hartley et al., 2005). Nevertheless, fresh- and brackish water bodies are found in the desert as well as hypersaline to saturated saltwater bodies occur in the desert itself and the Altiplano, the high plateau of the Andes (Demergasso et al., 2010, 2004; Dorador et al., 2013). High UV radiation, specific climatic and geological conditions have led to a fragmentation of habitable patches, which can only be occupied by organisms with specific adaptations (Arndt et al., 2020). Protists from different taxonomic groups including ciliates, placidids, percolomonads, choanoflagellates and also gregarines have been isolated and cultivated from the Atacama Desert (Arndt et al., 2020; Carduck et al., 2021; Rybarski et al., 2021; Schoenle et al., 2020).

We could isolate and cultivate ten *Cafeteria*-like strains from deep and surface waters of the North Atlantic (four strains), from brackish waters of the Baltic Sea (three strains), and from different salars in the Atacama Desert in Chile (three strains). Besides morphological characterization by high-resolution light microscopy and phylogenetic analyses of our *Cafeteria*-like strains by amplifying and sequencing several genes (18S rDNA, ITS-1, 28S rDNA), we exposed several strains to a wide range of salinities (2-150 PSU) to investigate their salinity tolerance.

Material and Methods

Sampling

Sampling of sediment and water samples for cultivation took place during two expeditions with the research vessel R/V Meteor (M139, M150) and one expedition with the research vessel Elisabeth-Mann-Borgese (EMB238) (Fig. 1, Table 1). Sediment samples were taken with a Multi-Corer system, while plankton samples were taken using a CTD-rosette system. Sediment slices from different layers were sampled. Subsamples of a few milliliters of the sediment suspension (filled up to 30 ml with autoclaved seawater) or water samples each of 30 ml were cultivated in 50 ml tissue culture flasks (Sarstedt, Nümbrecht, Germany). Water samples from three salars in the Atacama Desert were collected in 50 ml tubes (Sarstedt, Nümbrecht, Germany). Salinity was checked at each sampling position using a refractometer (HRS16, A.KRUSS Optronic, Hamburg, Germany). Isolation was carried out using a micromanipulator and/or microtiter plates. In total, ten *Cafeteria*-like strains from the different sampling regions were successfully cultivated (Fig. 1, Table 1).

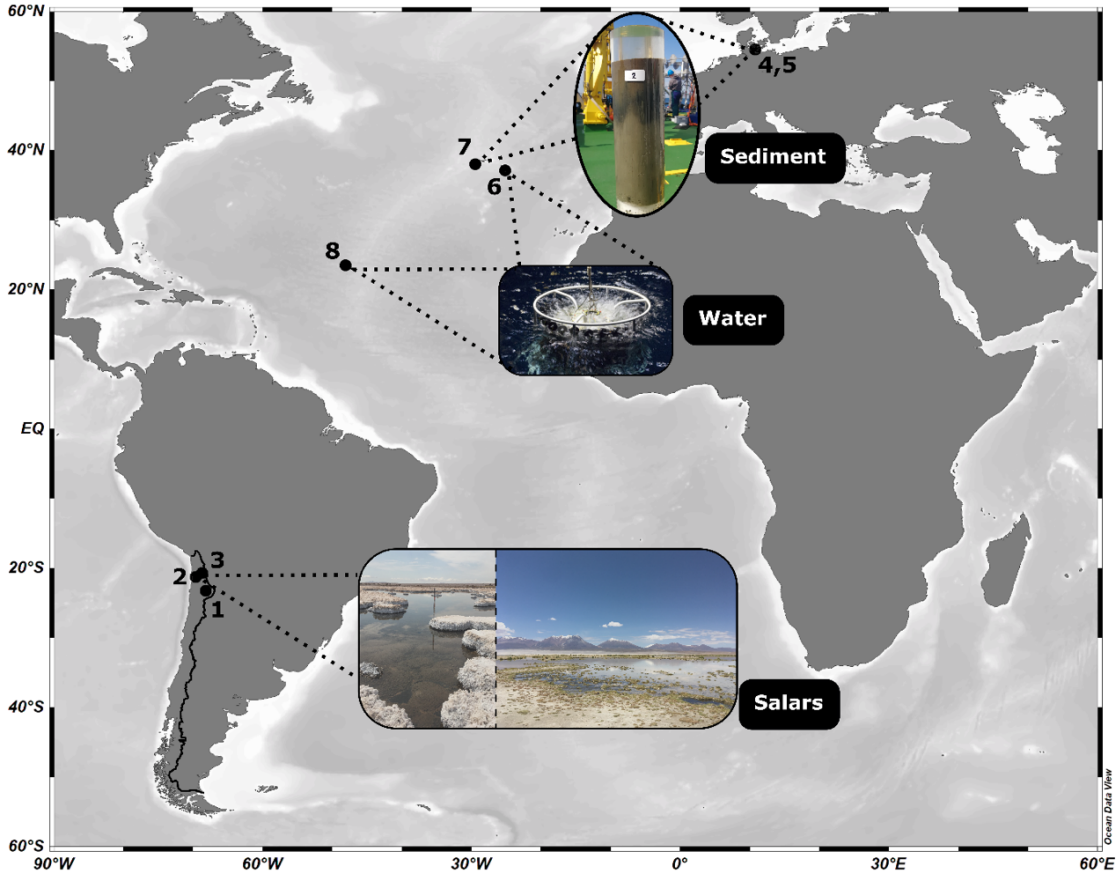


Figure 1. Sampling map of *Cafeteria*-like strains isolated from the marine realm (water and sediment sample, stations 4-8) and salars in the Atacama Desert (stations 1-3). Detailed information on sampling stations can be found in Table 1. The map was created using Ocean Data View (Schlitzer, 2012).

After isolation, the strains were cultivated in 50 ml tissue culture flasks (Sarstedt, Nümbrecht, Germany) filled with different media and salinities. The strains from the North Atlantic Ocean were cultivated in culture flasks filled with 30 ml Schmaltz-Pratt medium containing for 35 PSU per liter 28.15 g NaCl, 0.67 g KCl, 5.51 g $MgCl_2$ x 6 H_2O, 6.92 g $MgSO_4$ x 7 H_2O, 1.45 g $CaCl_2$ x 2 H_2O, 0.10 g KNO_3, 0.01 g K_2HPO_4 x 3 H_2O. For cultivation of strains from the Baltic Sea, Schmaltz-Pratt medium was diluted with autoclaved distilled water to 19 PSU. All cultures were supplied with sterilized quinoa or wheat grains as an organic food source for autochthonous bacteria. The Atacama strains were cultivated in artificial hypersaline water (AHW, 35 PSU / liter: 35.256 g NaCl, 0.886 g KCl, 4.43 g $MgCl_2$ x 6 H_2O, 0.21 g $MgSO_4$ x 7 H_2O, 0.151 g $CaCl_2$ x 2 H_2O)

and adapted to source salinity together with 10 % cereal grass solution as carbon source for bacteria. For the cereal grass stock solution, 1 g cereal grass (Lebepur GmbH, Berlin, Germany) was added to 1 litre of AHW medium with the required salinity, autoclaved and subsequently filtered. The stock solution was added to the AHW medium, diluted 1:10 and autoclaved again. The cultures were stored at room temperature and 13°C in the dark.

Table 1. Station list of marine *Cafeteria*-like strains collected and isolated during different deep-sea expeditions in the North Atlantic Ocean, one expedition in the Baltic Sea and from two expeditions to salars in the Atacama Desert, Chile.

HFCC No.	Depth/ Altitude [m]	Sampling gear	Sampling type	Sampling region	Station	Lat/Long	Year	Salinity [PSU]	18S rDNA (+ITS-1)	28S rDNA
Hyposaline and hypersaline inland waters (Atacama Desert, Chile)[1-3]										
865	2301	CT	water	[2] Salar de Atacama	Brine Shrimp Pool	23°17.300'S/ 68°10.433'W	2018	64	xxx ()	xxx ()
5012*	740	CT	water	[1] Salar de Llamará	Pump 2	21°16.200'S / 69°37.333'W	2019	5	xxx ()	xxx ()
5018	3729	CT	water	[1] Salar de Coposa	Point 3	20°40.684'S/ 68°41.717'W	2019	11	xxx ()	xxx ()
Hyposaline Baltic [4,5] R/V Elisabeth Mann-Borgese EMB238										
1637*	-23	MUC	Sediment (2-4 cm)	Baltic Sea	#5-5	54°32.77'N/ 10°46.61'E	2020	19	xxx ()	xxx ()
1645	-23	MUC	Sediment (6-10 cm)	Baltic Sea	#13-6	54°32.34'N/ 10°43.55'E	2020	19	xxx ()	xxx ()
1646	-23	MUC	Sediment (10-15 cm)	Baltic Sea	#13-6	54°32.34'N/ 10°43.55'E	2020	19	xxx ()	xxx ()
Deep Sea, Northern Atlantic[6,7] R/V Meteor M150										
937	-800	CTD	water	Santa Maria	#470, T14	37°10.011'N/ 25°11.185'W	2018	35	xxx ()	xxx ()
941	-800	CTD	water	Santa Maria	#470, T14	37°10.011'N/ 25°11.185'W	2018	35	xxx ()	xxx ()
1609	-2071	MUC	Overlaying water	Princess Alice Bank	#177, T5	38° 03.697'N/ 29° 25.627'W	2018	35	xxx ()	xxx ()
Marine surface waters, Northern Atlantic [8] R/V Meteor M139										
754	-250	CTD	water	NAO	#956	23° 33.23'N/ 48° 05.04'W	2017	35	xxx ()	xxx ()

Footnote 1 Superscripts behind ocean parts and sampling regions correspond to sampling stations indicated on the map (Fig. 1). Success of sequencing (18S rDNA (with ITS-1 marked with (x)), 28S rDNA) is indicated by accession numbers. HFCC: Heterotrophic Culture Collection Cologne; MUC: Multi-Corer; CT: Collection tube; Lat/Long: Latitude/Longitude. Several strains of the name-bearing types are in good condition and available as cultures at the manuscript submission date and are marked with an asterisk.

Light microscopy

For the morphological characterization, one representative strain of each of the three newly described species was analyzed, as reported within Schoenle et al. (2020). We used an Allen Video Enhanced Contrast (AVEC) system consisting of an analogous Hamamatsu C6489 camera with noise suppression and contrast amplification (Argus-20, Hamamatsu, Japan) to record high-resolution stills of the different strains. An inverted microscope (Zeiss Axio Observer A1) equipped with a 100x/1.4 NA oil immersion objective (DIC) and a water immersion condenser was used. Cultures were observed in Petri dishes with a coverslip base. Pictures were taken with VirtualDub-1.10.4 and were edited by ImageJ. Morphological characteristics (cell length, cell width, length of flagella) of specimens were measured with Axio Vision Rel. 4.8 (Zeiss, Germany).

Salinity tolerance

For long term cultivation, all strains were cultivated at 35 PSU. To ensure a similar growth phase, 5 ml of each strain were transferred into 50 ml tissue culture flasks (Sarstedt, Nümbrecht, Germany) filled with 25 ml artificial hypersaline water (AHW, 35 PSU). After seven days, 1 ml of each strain was inoculated into culture flasks (triplicates) filled with 20 ml AHW medium and heat-killed bacteria (*Pseudomonas putida* strain MM1) to ensure the food supply while changing the salinity. As a control, one culture flask of each strain without heat-killed bacteria was used. The salinity was stepwise increased (50 PSU, 75 PSU, 100 PSU, 125 PSU, 150 PSU) and decreased (25 PSU, 15 PSU, 10 PSU, 4 PSU, 2 PSU) every 48 h after checking for living cells. Strains exposed to each salinity were incubated and stored at 20 °C for seven days and then checked for moving cells (Fig. 2).

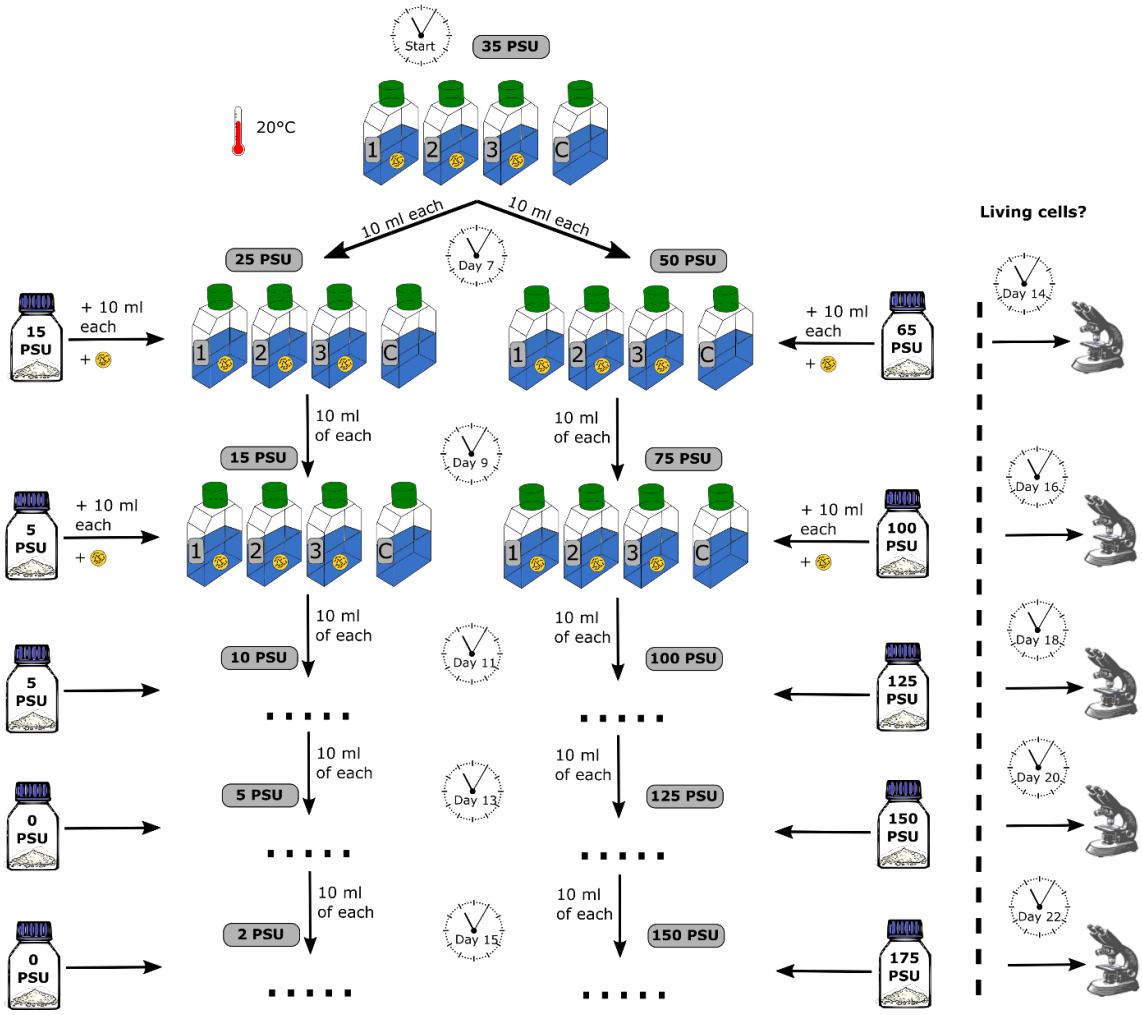


Figure 2. Experimental set-up for investigating the salinity tolerance of nine *Cafeteria* strains isolated from salars in the Atacama Desert (HFCC5012, HFCC5018), brackish waters in the Baltic Sea (HFCC1637, HFCC1645, HFCC1646) and the deep ocean (HFCC754, HFCC937, HFCC941, HFCC1609).

DNA extraction, amplification and sequencing

The DNA extraction, amplification and sequencing were conducted as described in Schoenle et al. (2020). The cultured strains were concentrated by centrifugation (4000 *g* for 20 min at 4°C, Megafuge 2.0 R, Heraeus Instruments). Genomic DNA was extracted using the Quick-gDNA™ MiniPrep (Zymo Research, USA). A long sequence from the 18S rDNA to the 28S rDNA was amplified with the primers 18S-For (5'-AACCTGGTTGATCCTGCCAGT-'3, (Medlin et al., 1988)) binding at the beginning of the 18S rDNA and NLR2098/24 (5'-AGCCAATCCTTWTCCCGAAGTTAC-3', (Van der Auwera et al., 1994))

binding at the end of the 28S rDNA. PCR reactions were performed in 25 µl PCR reaction mixtures containing 1.5 units (12.5 µl) TAQ (Red Taq DNA polymerase Mastermix, VWR Germany), 2 µl DNA and 2.5 µl of each primer (forward and reverse, concentration of 1 µM) at a final concentration of 0.1 µM. PCR conditions for amplifying the long products (18S-28S rDNA) were as follows: pre-denaturation at 98°C for 2 min, 35 cycles of 98°C for 30 s, 55°C for 45 s and 72°C for 4 min 30 s; final extension at 72°C for 10 min. Following internal primers were used for sequencing the 18S rDNA sequence, including the hypervariable V9-region located at the end of the 18S rDNA: 1280F (5'-TGCATGGCCGTTCTTAGTTGGTG-3', (Wylezich et al., 2002)) and 1389F (5'-TTGTACACCCGCCC-3', (Amaral-Zettler et al., 2009)). Additionally, the partial ITS-1 region was sequenced using the forward primer ITS-1 (5'-TAACAAGGTTTCCGTAGGTG-3', (Armbruster et al., 2000)). For several strains, a separate PCR amplification of the 28S rDNA had to be conducted with the forward NLF184/21 (5'-ACCCGCTGAAYTTAAGCATAT-3', (Van der Auwera et al., 1994)) and reverse NLR2098/24 primer. PCR conditions for amplifying the partial 28S rDNA were as follows: pre-denaturation at 98°C for 2 min, 35 cycles of 98°C for 30 s, 55°C for 45 s, 52°C for 45 s and 72°C for 2 min; final extension at 72°C for 10 min. The following internal primers were used additionally to sequence the 28S rDNA: the forward primers NLF1105/22 (5'-CCGAAGTTTCCCTCAGGATAGC-3', (Van der Auwera et al., 1994)) and D3-For (5'-GACCCGTCTTGAAACACGCA-3', (Wylezich et al., 2007)), and the reverse primer NLR1126/22 (5'-GCTATCCTGAGGGAAACTTCGG-3', (Van der Auwera et al., 1994)). The amplified products were analyzed by electrophoresis on 1% agarose gel, and fragment sizes were determined by comparison with 10,000 bp DNA ladder (Genaxxon Bioscience, Germany). The PCR products were purified by Fast Gene Gel/PCR Extraction Kit (Nippon Genetics, Düren, Germany). While 18S rDNA and 28S rDNA sequences were obtained for all strains despite HFCC5018 and HFCC865 due to extinction of the

cultures, the sequencing of the ITS-1 region was not successful for all strains. Thus, ITS-1 sequences were not included in our phylogenetic analyses but were deposited in GenBank for those strains, where sequencing of the ITS-1 region was successful.

Phylogenetic analyses

The phylogenetic analyses of *Cafeteria*-like strains were based on the 18S rDNA sequences of *Cafeteria* species, and other bicosoecids (including representatives of the genera *Caecitellus*, *Cafileria*, *Halocafeteria*, *Anoeca*, *Symbiomonas* and *Bilabrum*) retrieved from GenBank. Alignments were carried out using MUSCLE version 8.31 (Edgar, 2004) within UGENE version 1.37.0 (Okonechnikov et al., 2012); manual corrections to optimize the alignment were done with UGENE prior to the phylogenetic analysis, and the alignment was trimmed at both ends to a reasonable overlap. The 99-taxon alignment contained 1,735 well-aligned nucleotide positions that were used for the phylogenetic analyses. Alignments are available upon request. The Maximum likelihood (ML) analysis was carried out using RaxML-ng version 1.0.1 (Kozlov et al., 2019) on the CIPRES Gateway (Miller et al., 2010) employing the GTR (General Time Reversible) plus Γ model of nucleotide substitution and 40 random starting trees. Statistical support was estimated with 1,000 bootstrap replicates. Bayesian inference (BI) analysis was run with MrBayes v3.2.6 (Ronquist et al., 2012) using a GTR+I+Γ model and a four-category gamma distribution to correct among site rate variation. The search consisted of two parallel chain sets run at default temperatures with a sample frequency of 10 and run so that the average standard deviation of split frequencies dropped below 0.01. The analysis consisted of 3,000,000 generations, with a burn-in of 750,000 (25%), before calculating posterior probabilities.

For the concatenated tree, based on the 18S and 28S rDNA sequences, we used the *Cafeteria* alignment of Schoenle et al. (2020) and included eight of our

HFCC strains resulting in an alignment with 3,304 positions and 40 sequences. The Maximum likelihood (ML) analysis was carried out using MEGA X (Kumar et al., 2018), employing the GTR model. Statistical support was estimated with 500 bootstrap replicates using all sites of the alignment. Bayesian inference (BI) analysis was run with MrBayes v3.2.7 (Ronquist et al. 2012) using a GTR+I+Γ model and a four-category gamma distribution to correct for among site rate variation. The search consisted of two parallel chain sets run at default temperatures with a sample frequency of 10 and run so that the average standard deviation of split frequencies dropped below 0.01. The analysis consisted of 550,000 generations, with a burnin of 13,750, before calculating posterior probabilities.

For the calculation of the pairwise distances for the *Cafeteria* species we used MEGA X (Kumar et al., 2018) applying no variance estimation method and the pairwise deletion option. Pairwise distances of the *Cafeteria* species were calculated for each of the two phylogenetic trees (only 18S rDNA tree, concatenated tree based on 18S and 28S rDNA). Alignments were used, where sequences were cut to the same length. Phylogenetic trees were visualized by implementing the "ggtree" package (Yu, 2020) in R version 4.0.3 (R Core Team, 2020). The resulting trees were further prepared with Inkscape v0.92 (http://www.inkscape.org/).

ZooBank registration

ZooBank registration number of present work: xxxxx

Results

Ten strains of *Cafeteria*-like flagellates could be isolated, sequenced and morphologically described. The Atlantic strains could all be identified as *Cafeteria burkhardae*, while the Baltic strain and the strains isolated from the Atacama belonged to new species.

Light microscopy

All three species showed the same morphological and behavioural characteristics as described for the other *Cafeteria* species in Schoenle et al. (2020), including the orientation of the flagella and the overall shape (dorsal side mainly convex, ventral side flattened). Cells are attached to the substratum with the tip of the posterior flagellum. The anterior flagellum is directed upwards towards the right and used to catch bacteria. The main morphological differences between the examined strains are the overall shape of the cells and the formation of the shelf structure.

Description of new species

Stramenopiles Patterson, 1989 (emend. Adl et al. 2005)

Bicoecea Cavalier-Smith, 1993

Anoecales Cavalier-Smith, 1997

Cafeteriaceae Moestrup, 1995

Genus *Cafeteria* Fenchel and Patterson, 1988

***Cafeteria baltica* sp. nov. Hohlfeld, Sachs, Wiechmann, Arndt** (Fig. 3A-G)

Diagnosis: Free-living biflagellated bicosoecid, 3.2-4.9 μm long and 2.2-4.5 μm wide. Two flagella of about 3.9-6.9 μm length. D- to globular shaped cells. Species can tolerate freshwater to hypersaline conditions (2-125 PSU, sometimes up to 150 PSU).

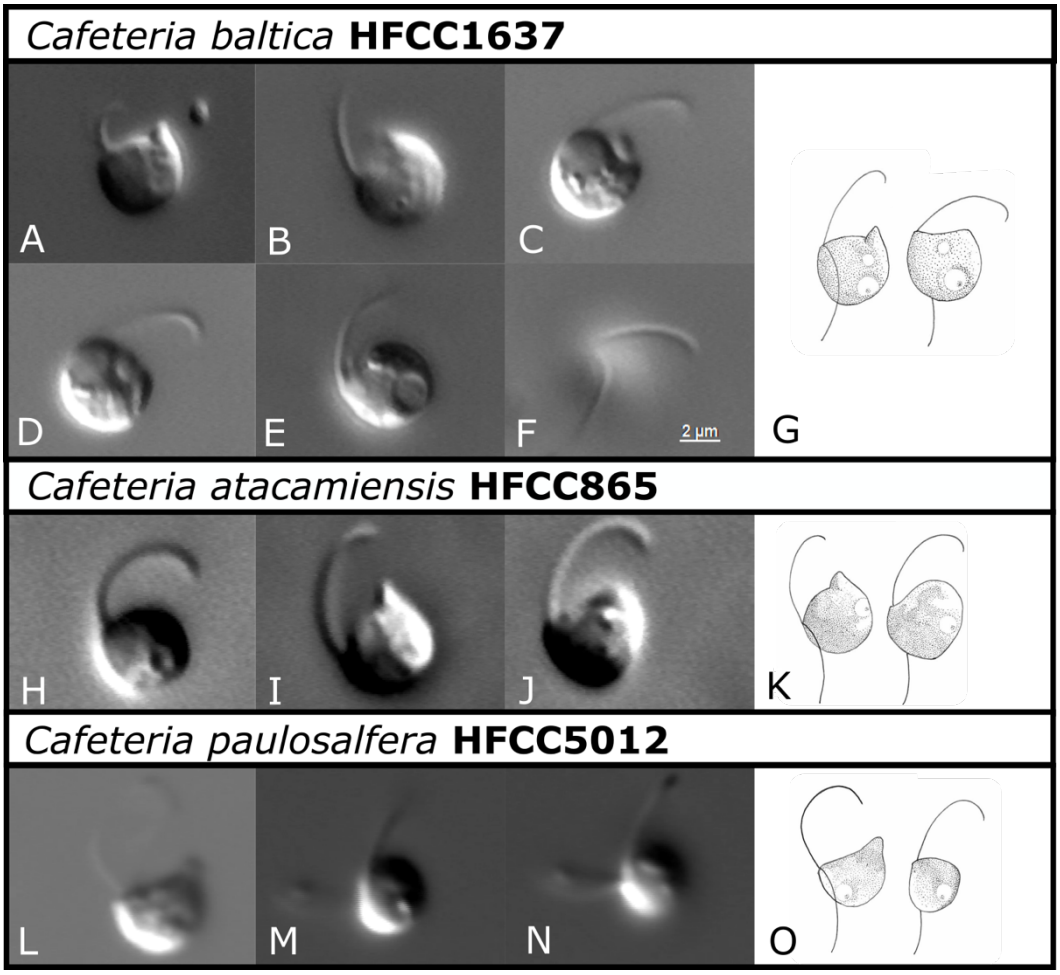


Figure 3. Differential interference contrast micrographs and drawings of *Cafeteria baltica* HFCC1637 (A-G), *C. atacamiensis* HFCC865 (H-K), *C. paulosalfera* HFCC5012 (L-O). Scale bar represents 2 µm.

Etymology: The species-group name *baltica* refers to the type locality (Baltic Sea).

Holotype: Specimen shown in Fig. 3A.

Type strain: Heterotrophic Flagellate Collection Cologne strain HFCC1637

Type sequence data: The 18S rDNA (+ITS-1) and 28S rDNA sequences have been deposited at GenBank with the Accession Numbers xxx and xxx.

Type locality: Littoral sediment layer (2-4 cm) at 23 m depths in the Baltic Sea (54°32.77'N and 10°46.61'E) at a salinity of 19 PSU.

ZooBank registration: will be provided

Description: Mean cell length with 4.0 ± 0.3 µm (3.2-4.9 µm, n=21) and mean cell width with 3.6 ± 0.4 (2.2-4.5 µm, n=21). Mean length of anterior flagellum of

equal length with 5.6 ± 0.7 µm (3.9-6.9 µm, n=19) as mean length of posterior flagellum length with 5.8 ± 0.8 µm (4.7-6.8 µm, n=7).

Remarks: *Cafeteria baltica* has the largest mean cell width with 3.6 µm, when compared to the other *Cafeteria* species described in Schoenle et al. (2020). The cells of *C. baltica* are D-shaped to globular as described for many of the *Cafeteria* species (Schoenle et al., 2020). The anterior flagellum is almost going straight up with a slight curve at the tip. The majority of *Cafeteria* species do hold their anterior flagellum in an arc form (e.g. *C. roenbergensis, C. burkhardae, C. biegae, C. maldiviensis, C. loberiensis*) except for *C. chilensis* holding it in a slight S-form or *C. graefeae* with no clear form (Schoenle et al., 2020). Flagella are of equal length in *C. baltica* and within the same size range as the flagella of *C. burkhardae* (Schoenle et al., 2020). The shelf of the cell is pronounced as observed for *C. atacamiensis*, *C. loberiensis* and *C. graefeae*. A pronounced furrow as observed in *C. burkhardae* was not visible. No extrusomes visible by light microscopy like in *C. chilensis*. Closest sequences in public databases (18S rDNA p-distance 0.4 %) belong to *C. burkhardae*.

***Cafeteria atacamiensis* sp. nov. Schoenle, Freches, Arndt** (Fig. 3H-K)

Diagnosis: Free-living biflagellated bicosoecid, 2.0-3.9 µm long and 1.7-3.2 µm wide. Posterior flagellum (3.7-11.5 µm) longer than anterior flagellum (3.2-6.4 µm). Cells round or roughly triangular shaped.

Etymology: The species-group name *atacamiensis* refers to the type locality (Salar de Atacama in the Atacama Desert, Chile).

Holotype: Specimen shown in 3J.

Type strain: Heterotrophic Flagellate Collection Cologne strain HFCC865

Type sequence data: The 18S rDNA sequence has been deposited at GenBank with the Accession Number xxx.

Type locality: Water from a brine shrimp pool at the Salar de Atacama in the Atacama Desert in Chile (23°17.300'S, 68°10.433'W) at a salinity of 64 PSU.

ZooBank registration: will be provided

Description: Mean cell length with 3.1 ± 0.4 µm (2.0-3.9 µm, n=26) and mean cell width with 2.6 ± 0.4 µm (1.7-3.2 µm, n=26). Anterior flagellum length with 4.9 ± 0.7 µm (3.2-6.4 µm, n=25) and posterior flagellum length with 7.3 ± 2.6 µm (3.7-11.5 µm, n=7).

Remarks: The anterior flagellum is either held in an arc form close to the cell body or slightly L-shaped, exceeding the anterior part. With its cell length and width *C. atacamiensis* belongs to the smaller fraction of *Cafeteria* species. The mean anterior flagellum length of *C. atacamiensis* is the second smallest of the so far described *Cafeteria* species. Only *C. graefeae* has a smaller mean anterior flagellum length as *C. atacamiensis*. The measurement of the posterior flagellum was not possible due to the extinction of the species in culture. The cell body of *C. atacamiensis* does not have the clear D-shaped form described, e.g. for *C. biegae, C. roenbergensis, C. maldiviensis* or *C. graefeae*, but is rather globular or roughly triangular shaped. Closest sequence in public databases (18S rDNA p-distance 3.9) belongs to MN315609 *C. maldiviensis*.

***Cafeteria paulosalfera* sp. nov. Rybarski, Schoenle, Arndt** (Fig. 3L-O)

Diagnosis: Free-living biflagellated bicosoecid, 2.2-3.8 µm long and 1.9-3.1 µm wide. Two flagella of unequal length; anterior flagellum about 3.2-7.7 µm length and posterior flagellum of 2.1-3.7 µm length. Slightly triangular to D-shaped cells.

Etymology: The species-group name *paulosalfera* refers to its low range of salinity tolerance. Paulo- (from paulus, latin = sparse), sal- (sal, latin = salt), fera (from ferendum, latin = tolerant). Species can tolerate freshwater to open ocean conditions (2-35 PSU).

Holotype: Specimen shown in 3L.

Type strain: Heterotrophic Flagellate Collection Cologne strain HFCC5012

Type sequence data: The 18S rDNA and 28S rDNA sequences have been deposited at GenBank with the Accession Numbers xxx and xxx.

Type locality: Water from the Salar de Llamará in the Atacama Desert in Chile (21°16.200'S, 69°37.333'W) at an altitude of 740 m and at a salinity of 5 PSU.

ZooBank registration: will be provided

Description: Mean cell length with 3.0 ± 0.4 µm (2.2-3.8 µm, n=20) and mean cell width with 2.5 ± 0.3 µm (1.9-3.1 µm, n=20). Anterior flagellum length with 5.2 ± 1.0 µm (3.2-7.7 µm, n=18) and posterior flagellum length with 2.8 ± 0.5 µm (2.1-3.7 µm, n=7).

Remarks: Regarding the mean cell width and length, *C. paulosalfera* lies in the smaller fraction of the *Cafeteria* species, together with *C. roenbergensis*, *C. maldiviensis* and *C. atacamiensis*. The posterior flagellum of *C. paulosalfera* is much smaller than observed for all the other *Cafeteria* species. The closest sequence in public databases (18S rDNA p-distance 1.7) belongs to MK177624 uncultured *Pseudobodo* sp. Compared to the other *Cafeteria* species, the closest *Cafeteria* sequence in public databases is *C. burkhardae* with a p-distance of 6.6%. Pairwise distance between *C. paulosalfera* HFCC5012 and *Cafeteria* sp. HFCC5018 (18S rDNA) is 0.4 %.

Salinity tolerance

All three investigated species were able to tolerate the salinity decrease to low saline waters. All *Cafeteria burkhardae* strains, isolated from the marine realm with 35 PSU, survived hypersaline salinities up to 150 PSU. The strains isolated from brackish waters in the Baltic sea, *C. baltica* (original salinity ~19 PSU), survived until 125 PSU, except for one strain (HFCC1645), which showed survival until 150 PSU. The two strains belonging to *C. paulosalfera*, isolated from Salars in the Atacama Desert, only showed survival until max. 50 PSU, while their salinity of source material was 5-11 PSU. Due to the early extinction

of strain HFCC865 (*C. atacamiensis*) in culture, we were not able to conduct salinity experiments with this strain.

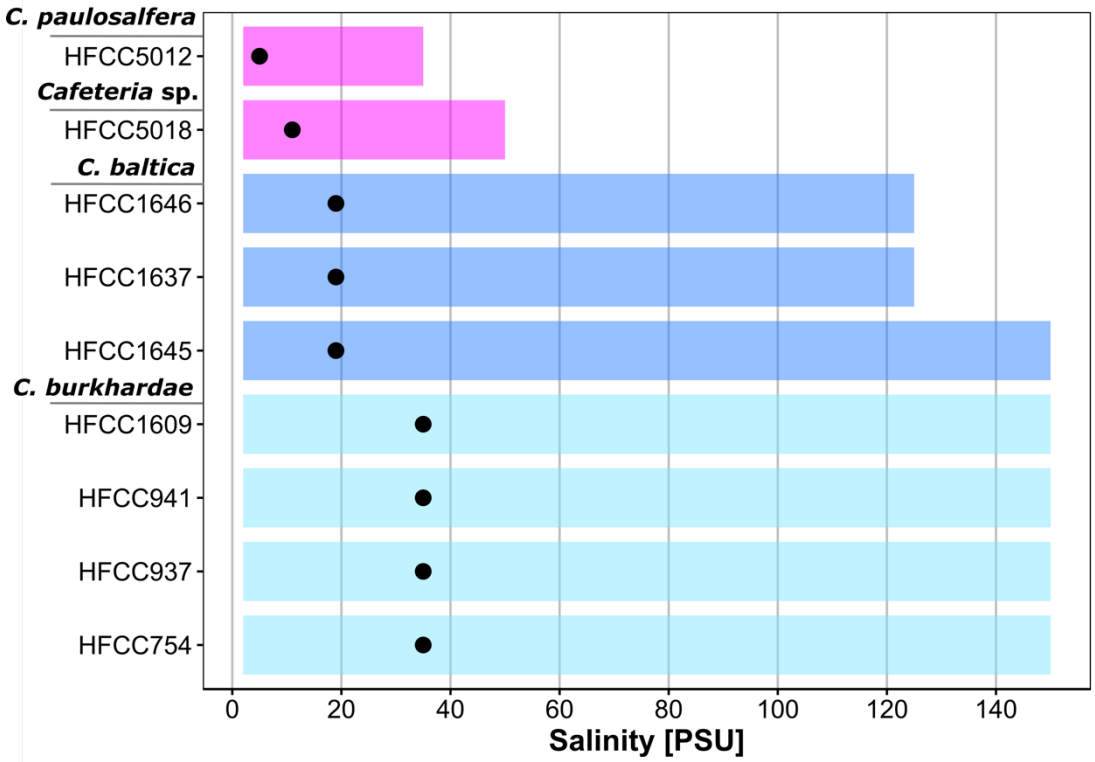


Figure 4. Salinity tolerance ranges (from 2 to 150 PSU) of nine *Cafeteria* strains isolated from salars in the Atacama Desert (HFCC5012, HFCC5018), brackish waters in the Baltic Sea (HFCC1637, HFCC1645, HFCC1646) and the Atlantic (HFCC754, HFCC937, HFCC941, HFCC1609). Bars indicate moving cells. Dots show the original salinity of the source material of each strain.

Phylogenetic analysis

As our phylogenetic analysis aimed to analyse the placement of the newly isolated *Cafeteria* strains and newly described *Cafeteria* species, we did not include all bicosoecid groups in our 18S rDNA analysis. We decided to focus on the Anoecales and only included the genus *Cafileria* and *Halocafeteria* besides sequences from *Cantina marsupialis* and MAST-24, which were used as outgroup. Anoecales included the families of Cafeteriaceae, Caecitellaceae, Symbiomonadaceae, Anoecaceae and the genus *Bilabrum* (Fig. 5B). The recently described species *Cafileria marina* was included in our analysis due to its seemingly close relationship to *Caecitellus*. However, in our study, *Cafileria* did

not form a cluster with *Caecitellus*, but instead clustered outside the Anoecales. Although the cluster was moderately/highly supported (mlBP 84, biPP 1.0) one must keep in mind that we did not include all bicosoecid groups and that the resolution of the bicosoecids is still an ongoing issue. *Halocafeteria* formed a branch with low support by bootstrap probability but high Bayesian inference posterior probability (mlBP 68, biPP 1.0). The outgroup consisting of *Cantina marsupialis* and MAST-24 formed a high supported cluster (mlBP 92, mlBP 1.0). *Bilabrum latius* clustered outside of Anoecales. The Cafeteriaceae clade was highly supported (mlBP 98, biPP 1.0). Almost all species within the Cafeteriaceae showed a moderate to high phylogenetic support. Pairwise distances within the *Cafeteria* species based on the 18S rDNA ranged from 0.4% (*C. burkhardae* to *C. baltica*) to 7.9% (*C. paulosalfera* to *C. atacamiensis*) (Fig. 5A).

The concatenated analysis of the 18S- and partial 28S rDNA together with *Caecitellus* as outgroup resulted in a highly resolved tree, with most branches having bootstrap support of 100 (mlBP) or 1.0 (biPP) (Fig. 6B). Tree topology for the Cafeteriaceae was similar to the 18S rDNA topology. *Cafeteria atacamiensis* could not be included in the concatenated tree due to the extinction of the species in culture and missing 28S rDNA sequence. Pairwise distance measurements of the concatenated alignment of the *Cafeteria* type strains (Fig. 6A) showed an increase in almost all p-distances within a range from 1.3 % (*C. graefeae* HFCC33 to *C. loberiensis* HFCC843) to 3.4 % (*C. paulosalfera* HFCC5012 to *C. mylnikovii* CCAP1900/2). The p-distances between *C. baltica* HFCC1645 to *C. burkhardae* HFCC168 and *C. loberiensis* HFCC843 to *C. chilensis* HFCC832 stayed the same (0.4 % and 0.5 %, respectively). Including the 28S rDNA into the p-distance analysis resulted in a p-distance decrease of 0.1 % from *C. mylnikovii* to *C. chilensis*/*C. loberiensis*.

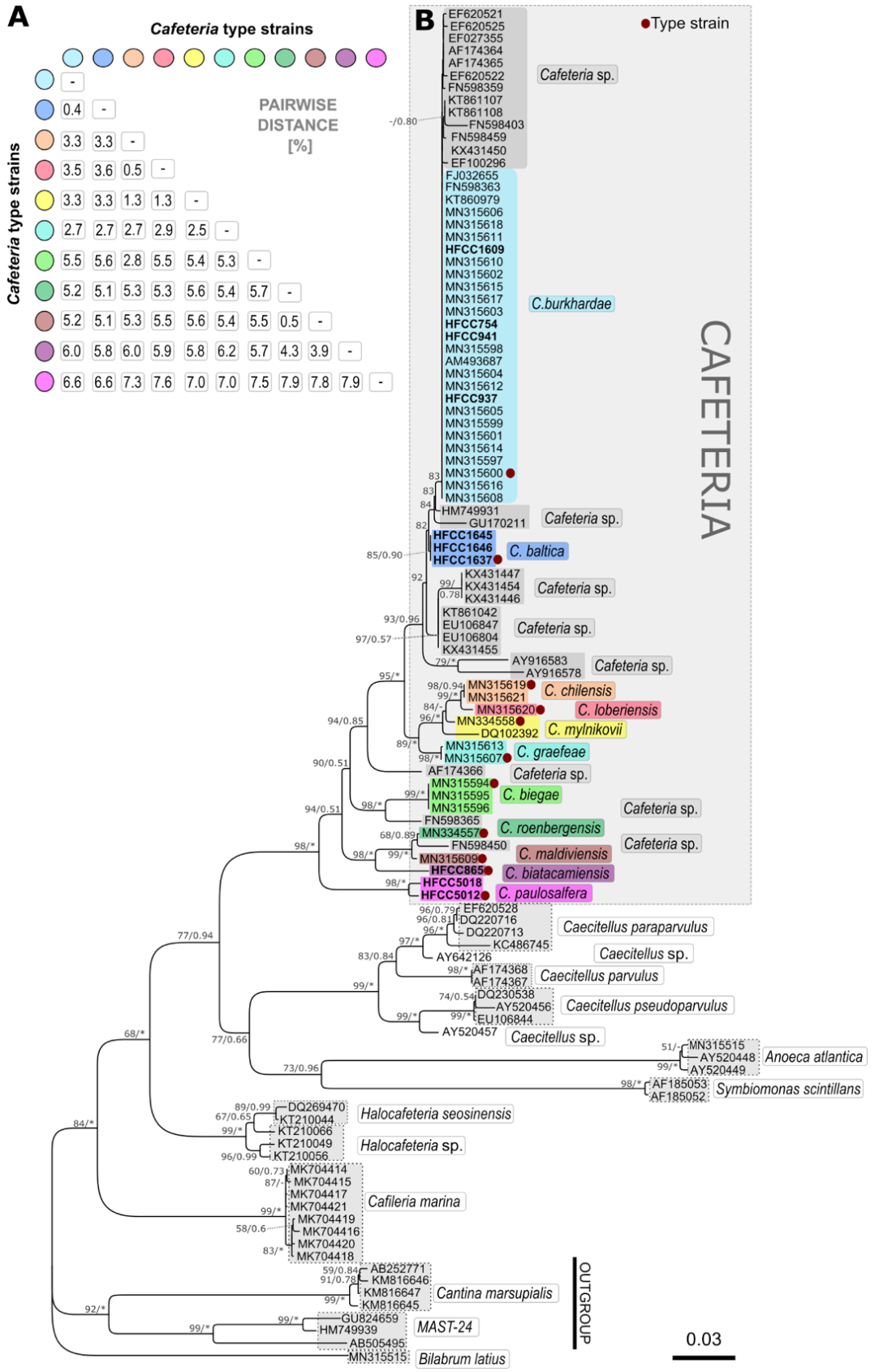


Figure 5. Bayesian phylogenetic tree of several bicosoecid groups based on 18S rDNA sequences. Branches receiving 100% maximum likelihood bootstrap percentages (mlBP; GTR+Γ model, 1,000 replicates) and 1.00 Bayesian inference posterior probability (biPP) support are denoted by an asterisk, values are otherwise given at branches, values <50% are not shown. Scale bar (right bottom) represents 0.03 expected substitutions/site in the ML analysis. The tree is outgroup-rooted, with *Cantina marsupialis* and MAST-24 as outgroup.

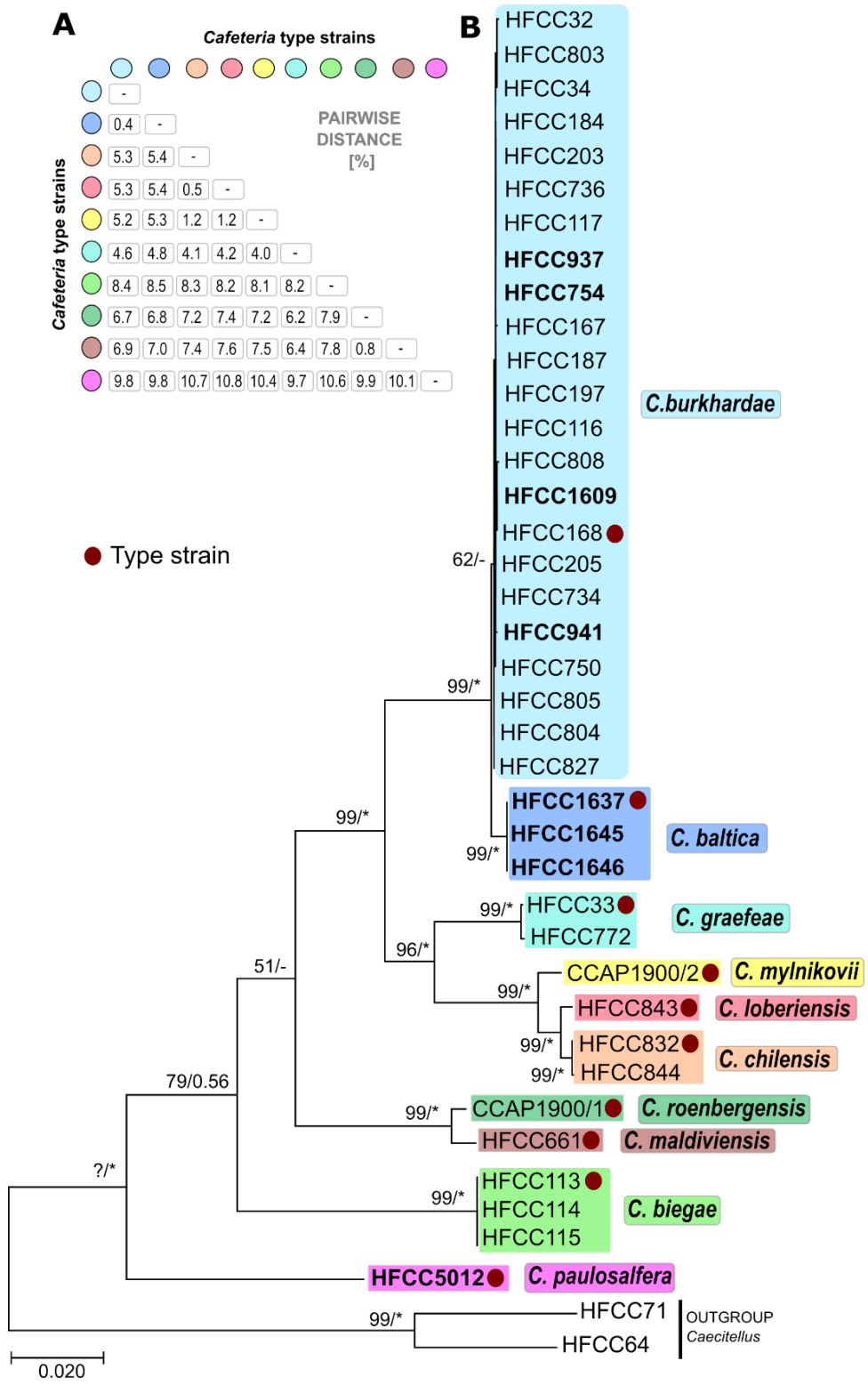


Figure 6. Maximum likelihood phylogeny (ML; GTR+Γ model, 500 replicates) of the Cafeteriaceae based on a concatenated dataset of 18S- and partial 28S rDNA, 3,304 aligned nucleotide positions. The tree is outgroup-rooted with *Caecitellus*. A Bayesian Inference (BI) under the same model yielded an identical topology. Branches receiving 100% maximum likelihood bootstrap percentages (mlBP) and 1.00 Bayesian inference posterior probability (biPP) support are denoted by an asterisk, values are otherwise given at branches. Values lower than 50 (mlBP) or 0.5 (biBP) are not shown and indicated with '-'. Scale bar (bottom) represents 0.02 expected substitutions/site in the ML analysis.

Discussion

Morphological characteristics obtained by high-resolution light microscopy of *Cafeteria* species reveal only small differences between the species described within this study and a previous study (Schoenle et al., 2020). Thus, we included molecular and autecological characteristics in our integrative taxonomic approach, as already suggested and conducted for protists (Boenigk et al., 2012; Carduck et al., 2021; Schiwitza et al., 2018). Due to the high difference in salinities within the inhabiting environments (brackish waters, open ocean and hypersaline salars), we conducted salinity experiments to investigate the salinity tolerance of all strains. Besides the presence of the common *Cafeteria burkhardae*, we discovered three new species, two from salars located in the Atacama Desert (*C. paulosalfera, C. atacamiensis*) and one from the Baltic Sea (*C. baltica*). All *Cafeteria* species have a shelf structure, which is distinctly pronounced, while only some species have a pleat like thickening around the ventral side of the cell (Table 2). Comparison of morphological measurements of all so far morphological and genetical described *Cafeteria* species (Fig. 7, Table 2) revealed only in a few cases (cell width of *C. baltica* HFCC1637 and cell length of *C. loberiensis* HFCC843) significant differences to all other *Cafeteria* species (Fig. 7 Tukey's post-hoc test).

The phylogenetic analysis of the 18S rDNA and the concatenated tree (18S and 28S rDNA) yielded trees with identical topologies, while *Cafeteria* clades were supported by higher bootstrap support within the concatenated tree. Including more genes in the phylogenetic analysis resulted in a higher robustness of the tree and topology of the *Cafeteria* clades, as has been shown in previous studies (Brown et al., 2013; Pánek et al., 2015; Carr et al., 2017; Lax et al., 2021). Phylogenetic analyses of our newly isolated strains and the so far described *Cafeteria* species showed clear species-level clades differing by more than 2.5 % of the 18S rDNA for the majority of *Cafeteria* species. When including the 28S rDNA, the genetic distance increased in almost all cases. One of the

exceptions are *Cafeteria baltica* and *C. burkhardae,* which are genetical closely related when comparing the 18S and 28S rDNA (p-distance of 0.4 %). However, the ITS-1 region of these two species differed highly from one another, increasing the molecular differences between these two species up to a p-distance of 4.1 % (18S rDNA + ITS-1 region). Thus, we described the three strains isolated from the Baltic Sea as new species, namely *C. baltica*. Although the original salinity of isolation of *C. baltica* was around ~19 PSU in the Baltic Sea, all three isolated strains were able to survive salinities up to 125 PSU at least. However, overall *C. burkhardae* coped slightly better with high salinities up to 150 PSU than *C. baltica* in our salinity tolerance experiments. *Cafeteria burkhardae* has been identified by cultivation and next-generation-sequencing techniques as a widely distributed, and abundant species in the marine realm, from surface waters down to the deep sea (de Vargas et al., 2015; Massana et al., 2020; Schoenle et al., 2020). *Cafeteria burkhardae* has been shown to survive high hydrostatic pressure up to 550 bar indicating the potential to thrive in the deep sea (Živaljić et al., 2018). The high salinity tolerance of *C. burkhardae* observed within this study might indicate the potential to occupy more extreme habitats in regards of salinity besides the open ocean, underlying its potential for a cosmopolitan distribution.

Strains isolated from the Atacama Desert have a high molecular distance (18S rDNA) to all the other *Cafeteria* species, e.g. *C. paulosalfera* HFCC5012 with a p-distance >6.6 % and *C. atacamiensis* HFCC865 with a p-distance >3.9 %. Thus, we assigned these strains to new species. Due to the early extinction of *C. atacamiensis* HFCC865 in culture, no salinity tolerance experiments and sequencing of the 28S rDNA could be conducted. *Cafeteria paulosalfera* (HFCC5012) originates from groundwater being flushed into the Salar and did not survive high salinities (≥35 PSU) during our experiments. Strain HFCC5018 was isolated from the Salar de Coposa with maximum reported salinities of 40 PSU and survived until ≥50 PSU. Comparing the 18S rDNA of those two strains

showed, that they differed by 7 bp in a 1580 bp long sequence (p-distance 0.4 %). This p-distance (18S rDNA) is the same as reported for *C. baltica* and *C. burkhardae*. However, we could not recover the ITS-1 region or 28S rDNA for strain HFCC5018, which would give insight if strain HFCC5018 either belongs to *C. paulosalfera* or is a completely different species. We decided to run HFCC5018 as separate species (*Cafeteria* sp.) until further sequencing has been conducted. Unfortunately, the culture of *Cafeteria* sp. strain HFCC5018 is contaminated and re-isolation of HFCC5018 was not successful. Thus, resampling at this point at the Salar de Coposa would need to be conducted to try to get HFCC5018 again in culture. Overall, hyperarid conditions of the Atacama Desert in concert with high UV radiation lead to isolation and radiation of organisms by spatially separating populations for different periods of time (Arndt et al., 2020).

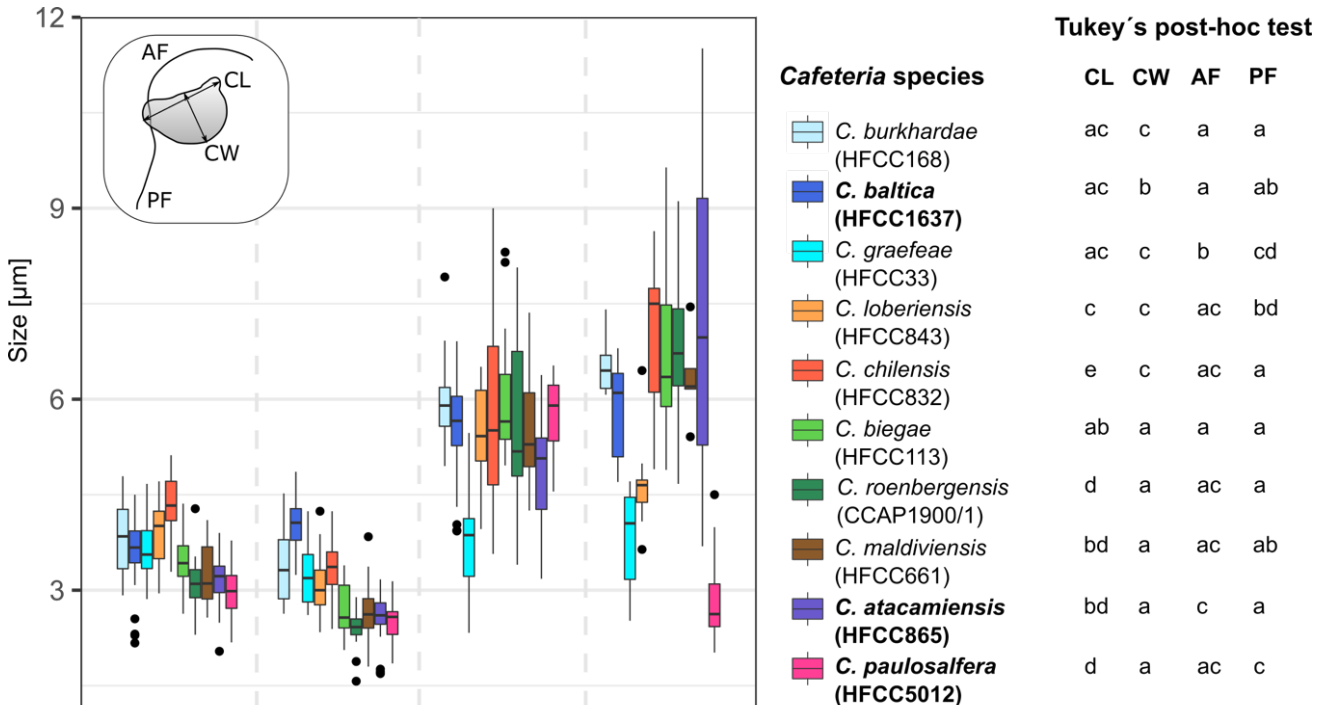
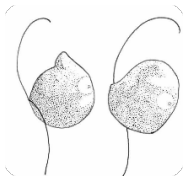
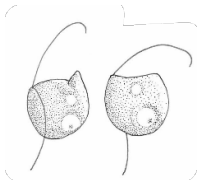
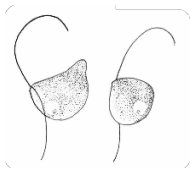
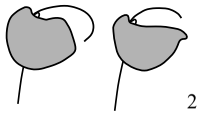
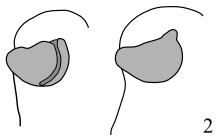
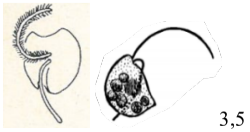
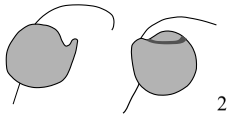
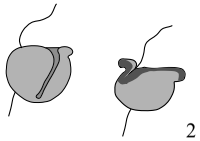
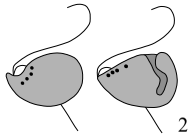
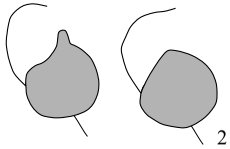


Figure 7. Boxplots of morphological measurements of newly (names in bold within the legend) and already described *Cafeteria* species. Measurements of morphological features included cell length (CL), cell width (CW), anterior flagellum (AF) and posterior flagellum (PF) indicated in the scheme in the upper left of the graph. An ANOVA was used to compare morphological measurements of the different species. Different letters indicate significant ($p < 0.05$) differences between measurements (Tukey´s post-hoc test).

Table 2. Main morphological characteristics of all so far described *Cafeteria* species. CL: cell length, CW: cell width, AF: anterior flagellum, PF: posterior flagellum.

CL [µm] mean ± sd	CW [µm] mean ± sd	AF [µm] mean ± sd	PF [µm] mean ± sd	Cell shape (CS)	Shelf structure (SSt)	Furrow (F)/ Pleat-like thickening (PLT)	Channel (CH)	Flagella		Occurrence	Described by (A-H) / references (1-6)
C. biatacamiensis **(HFCC865)**											
3.1 ± 0.4	2.6 ± 0.4	4.9 ± 0.7	7.3 ± 2.6	- globualar or roughly triangular shaped	yes pronounced	no / no	no	AF in arc form close to cell body or slightly L-shaped, exceeding anterior part		Salar de Atacama in the Atacama Desert, Chile	A / 1
C. baltica **(HFCC1637)**											
4.0 ± 0.3	3.6 ± 0.4	5.6 ± 0.7	5.8 ± 0.8	- D-shaped/globular - ventrally flattened	yes pronounced	no / no	no	AF almost straight with slight curve at the tip		litoral sediment at the Baltic Sea	B / 1
C. paulosalfera **(HFCC5012)**											
3.0 ± 0.4	2.5 ± 0.3	2.8 ± 0.5	5.2 ± 1.0	- slightly triangular to D-shaped cells - right side more or less roundish shaped	yes pronounced	no / no	no	AF in hoes form		Salar de Llamará in the Atacama Desert, Chile	C /1
C. maldiviensis **(HFCC661)**											
3.3 ± 0.5	2.7 ± 0.6	5.5 ± 1.5	4.8 ± 3.0	- D-shaped - left anterior part truncated - ventrally flattened	yes	no / no	no	AF in arc form close to cell body, not exceeding anterior part	 2	litoral sediment (Maldive Islands)	D / 2
C. biegae **(HFCC113)**											
3.5 ± 0.5	2.7 ± 0.6	5.9 ± 1.4	6.7 ± 2.2	- D-shaped - laterally ventral depressed - right side more or less roundish shaped	yes pronounced	PLT from SSt at anterior ventral side to middle posterior front,	curved towards dorsal middle side, almost reaches dorsal side going straight up	AF in arc form, exceeding anterior part	 2	deep-sea sediment (Mediterranean Sea)	E / 2
C. roenbergensis **(CCAP1900/1)**											

3.1 ± 0.4	2.4 ± 0.4	5.6 ± 1.4	6.8 ± 1.6	- D-shaped - flat unembellished ventral surface - shallow groove on the left side	yes	no / no	no	AF in arc form of about 180°, extends laterally	 3,5	surface waters at the Limfjord in Jutland, Denmark	F / 2, 3, 4
C. burkhardae **(HFCC168)**											
3.1 ± 0.4	3.4 ± 0.8	5.8 ± 1.4	5.9 ± 2.2	- D-shaped/globular - ventrally flattened	yes	concave ventral F surrouned by ovoid PLT	no	AF in arc form, exceeding anterior part	 2	cosmopolitan distributed in marine realm, surface waters down to deep sea	E / 2
C. graefeae **(HFCC33)**											
3.7 ± 0.5	3.2 ± 0.8	3.9 ± 1.0	3.9 ± 0.9	- D-shaped - laterally ventral depressed	yes pronounced	yes /yes	curved from ventral F towards dorsal middle side of the cell	AF almost straight and with no clear form	 2	surface waters and deep-sea sediment in Atlantic Ocean	E / 2
C. chilensis **(HFCC832)**											
4.4 ± 0.5	3.3 ± 0.8	6.0 ± 1.7	7.0 ± 1.7	- raindrop shaped - extrusomes along right margin of flagellar depression in ordered rows	no	no	curved in S-form at posterior end reaching dorsal side of cell.	AF curved in slight S-form close to cell body	 2	rockpool in Chile	G / 2
C. loberiensis **(HFCC843)**											
3.3 ± 0.10	3.0 ± 0.7	5.4 ± 1.4	4.4 ± 1.6	- D-shaped/globular - right side more or less roundish shaped	yes pronounced	no / yes (2x) two PLTs: one reaching from SSt at anterior front of ventral side to middle of the posterior front and other parallel to first PLT closer to doral side	no	AF in arc form	 2	rockpool in Chile	G / 2
C. mylnikovii **(CCAP1900/2)**											
3-5	NA	6-10	~5	- D-shaped - laterally compressed	yes	yes / yes	NA	AF in arc form	NA	White Sea coast, near village of Kartesh, Russia	H / 6

Footnotes: NA = Not available

Described by

A Schoenle and Arndt, 2021
B Hohlfeld and Sachs, 2021
C Schoenle and Rybarski, 2021
D Schoenle, Rosse and Arndt, 2020
E Schoenle and Arndt, 2020
F Fenchel and Patterson, 1988
G Filz, Nitsche and Arndt, 2020
H Cavalier-Smith and Chao, 2006

References

1 this study
2 Schoenle et al. 2020
3 Fenchel and Patterson 1988
4 Al-Qassab et al. 2002
5 Larsen and Patterson 1990
6 Cavalier-Smith and Chao 2006

Author contributions

All authors were involved in the sampling and cultivation of protists. M.H., A.R., C.M, P.S. and E.F. conducted the molecular and morphological laboratory investigations. A.R., A.S. and M.H. conducted the salinity experiments of the strains. M.S. conducted the drawings of the *Cafeteria* species. A.S. performed the phylogenetic analyses and wrote the first version of the manuscript. All authors reviewed the manuscript.

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Part 2

Biodiversity and distribution of aquatic protists in extreme environments of the Atacama Desert

Chapter 4

High diversity and isolated distribution of aquatic protists in salars of the Atacama Desert at extremely high salinities

High diversity and isolated distribution of aquatic protists in salars of the Atacama Desert at extremely high salinities

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Abstract

At extreme hypersaline conditions, the species richness of eukaryotes is generally assumed to be low. On the other hand, various studies showed a high degree of phylogenetic novelty at these extreme conditions. Also, the variation of chemical parameters in extreme habitats may influence the eukaryotic community structure. These findings call for a more thorough look into the species richness of hypersaline environments, which seems to be more diverse than previously thought. In this study, various hypersaline lakes as well as further aquatic ecosystems of northern Chile were investigated regarding protistan species diversity and richness by metabarcoding studies of environmental samples. Moreover, heterotrophic flagellates from various water bodies were isolated for detailed molecular, morphological, and ecological investigations. The comparison of metabarcoding studies together with the studies of isolated protist cultures from the different extreme habitats gave us information on the biodiversity and distribution of protists. In addition, the results were used to analyse global distribution patterns of the recently discovered class Placididea (Stramenopiles) which was used here also as a model group for the study of protist distribution in hypersaline environments. To relate taxa composition to the abiotic environment, the chemical composition of these salar lakes was determined by Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES). Even though relative ion concentrations were relatively similar, investigations of genotypes showed a unique community composition in nearly each salar and even among different microhabitats within one salar. The relatively low dispersal rate and the ability of many protist species for a relatively fast genetic adaptation at high generation times suggests that the salars should be considered as hot spots of protist evolution, especially for representatives of Stramenopiles and Alveolata.

Keywords: Placididea; V9 SSrDNA; Hypersaline waters; eDNA; Evolution; Water chemistry; Stramenopiles; Heterotrophic flagellates

Introduction

Hypersaline environments are thought to be characterized by a low species richness, especially at extremely high salinities (Pedrós-Alió, 2004). However, various recent studies on unicellular organisms have also shown a high degree of phylogenetic novelty under these extreme conditions (Demergasso et al., 2004; Dorador et al., 2013; Triadó-Margarit and Casamayor, 2013; Eissler et al., 2019; Dorador et al., 2020; Lee et al., 2021) including representatives of all taxonomic groups like archaea, bacteria, viruses, and eukaryotes (Emerson et al., 2013; Oren, 2014). In unicellular eukaryotes, the novelty can be assigned to species that primarily belong to the supergroups of Stramenopiles (Bicosoecida) or Opisthokonta (Choanoflagellata) (Triadó-Margarit and Casamayor, 2013) and previous studies have identified several new species isolated from hypersaline environments showing special adaptation to high ranges of salt concentrations (Park and Simpson, 2010; Schiwitza et al., 2018; Rybarski et al., 2021; Heine-Fuster et al., 2021; Schoenle et al., in prep). Further species from the group of Discoba (Heterolobosea) (Tikhonenkov et al., 2019; Carduck et al., 2021) and Alveolata (Ciliophora) (Qu et al., 2020) were also isolated successfully from these environments. These findings called for a more thorough look into the biodiversity of hypersaline environments, which are more diverse than previously thought (Harding and Simpson, 2018). The northern part of Chile is one place where salars or salt flats are particularly common (Risacher et al., 2003). They are separated by the Coastal Cordillera and the Precordillera of the Andes and located in the Central Depression and the Western Cordillera in the Altiplano (≥ 3800 m a.s.l). The special position of the salars as isolated and its surrounding by almost sterile habitats (Azua-Bustos et al., 2012; Neilson et al., 2012; Valdivia-Silva et al., 2012) makes them interesting locations with potentially highly adapted organisms and unique biodiversity (Warren, 2006). Organisms at these locations must not only be adapted to high salinities but also to high temperatures, high evaporation rates,

high altitude, and high UV radiation. The presence of arsenic and lithium as toxic metalloids could also affect organisms and may play a role in species composition (Volant et al., 2016) and also the variation of chemical parameters may influence eukaryotic community structure (Mo et al., 2021). As potentially fast-evolving organisms with a high reproduction rate and the ability to adapt quickly to extreme conditions, protists in these extreme environments are particularly suitable for investigations on evolutionary processes (Arndt et al., 2020). Before the Atacama region developed, placidids (Stramenopiles) evolved from marine representatives (131 (77–196) Ma ago) and their separation from marine and hypersaline occurred around 114 (66-175) Ma ago (Arndt et al., 2020). Multiple strains of placidid species were isolated from various hypersaline environments in the Atacama Desert (Rybarski et al., 2021), also different species from one salar, which suggests allopatric as well as sympatric divergence processes with synchronous diversification. Placidids seem to be highly adapted to these extreme and isolated habitats and may be used as a model group to characterize these environments (Arndt et al., 2020). The formation of resting stages (cysts) enables them to be transported into new habitats, e.g. via wind or animals like birds (Rogerson and Detwiler, 1999), and to survive changes to non-optimal conditions. However, the exchange rate seems to be low since most salars are hundreds of kilometres apart and in between, there is extremely high UV radiation, and mostly stepping posts are lacking. It can thus be hypothesized that although there is a small exchange between the different salars, e.g. via birds, this exchange occurs very seldom, supporting evolutionary processes by isolation. On the other hand, the high diversity of microhabitats in the same salar might allow the coexistence of many genotypes. Do each salar have its unique eukaryotic community that has been specifically adapted? To get a better overview of the hidden protistan diversity and richness in the Atacama Desert, the V9 region (SSU rDNA) of environmental samples from 11 isolated inland waters (hypersaline salars,

lagoons, lakes) located at different altitudes with different salt concentrations was investigated. To keep sequencing errors and the overestimation of taxa richness to a minimum, a mock community as a reference community was used. These filter results were also compared to the filtering carried out in other metabarcoding studies for comparison (e.g. Schoenle et al., 2021). In addition to the metabarcoding approach, a culture-based approach was accomplished to detect the occurrence of species and to add verified sequences to the V9 region data base using morphological and molecular tools. Where possible, samples from one location but from separated pools with different salinities were additionally taken, to find out if each location contains unique eukaryote communities or whether there is a large overlap and potential exchange between sampling sites.

Material and Methods

Sampling and DNA isolation of filter samples

The classification of inland water samples regarding their salinity was made according to Hammer (1986). Samples with a salinity of 0.5-3 PSU (practical salinity units) were considered subsaline, 3-20 PSU hyposaline, 20-50 PSU mesosaline and > 50 PSU hypersaline. Surface water was filtered from various inland waters with different salt concentrations from northern Chile during several expeditions (Table1, Figure 1). A suction filter holder (Sartorius Japan) with a polycarbonate filter (Sartorius, 47 mm, 0.45 µm) was used for the filtration. The amount of filtered water for each sample can be found in Table 1. Filters were transferred immediately into CryoTubes™ (Thermo Fisher Scientific, Waltham) and covered completely with 2 ml of ethanol (samples from 2017, 2019) or with a DMSO/EDTA/NaCl solution (DESS) for preservation (samples from 2018) (Gray et al., 2013; Yoder et al., 2006). At every location, an additional water sample was taken to identify chemical parameters, such as the composition and concentration of different ions. Parameters, like the exact geographic location together with the

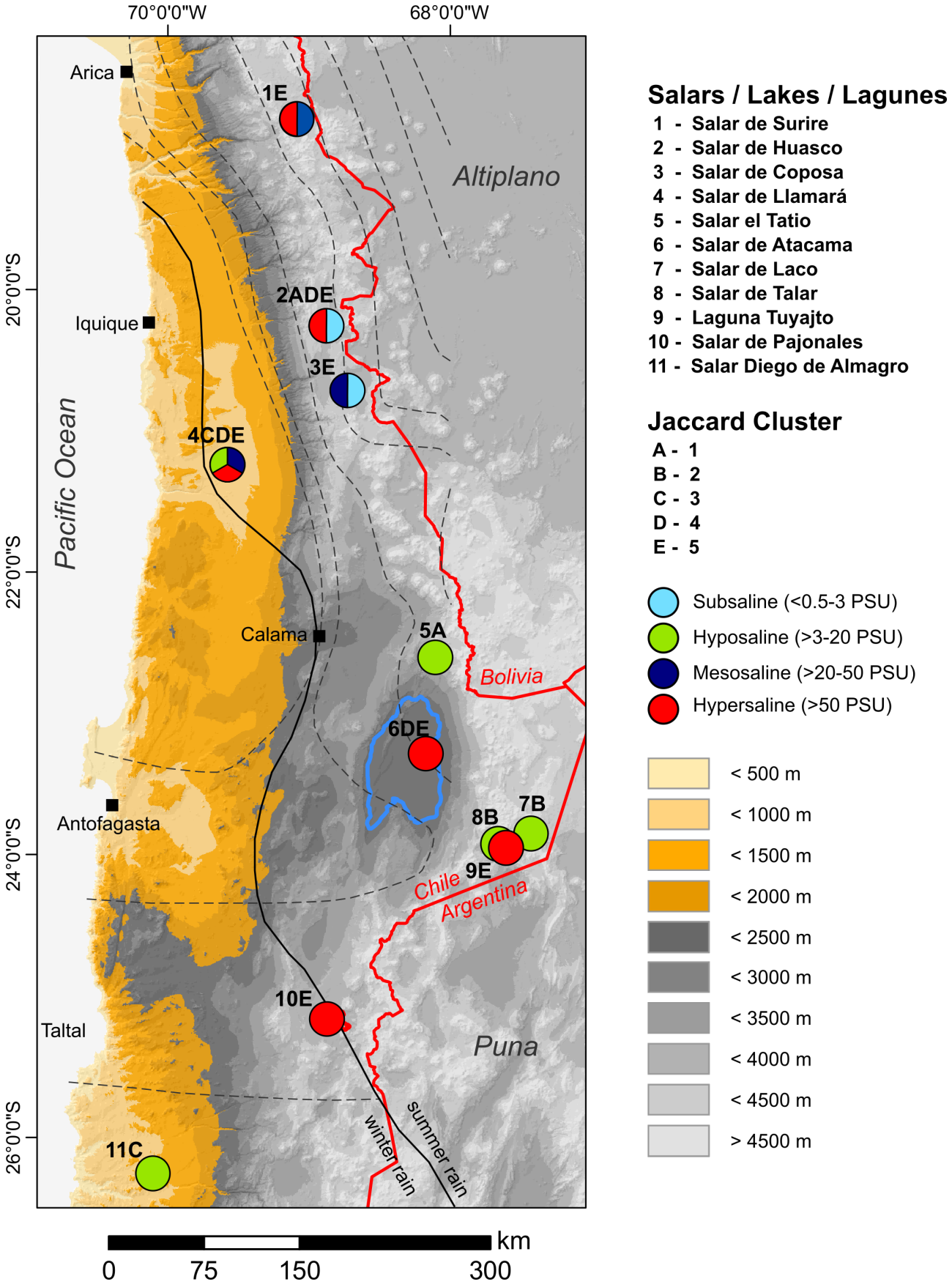

Figure 1. Map of sampling points in northern Chile. Colours of the positions indicate their salinity, several colours at one position demonstrate multiple samples with different salinities. Letters indicate the resulting cluster of the Jaccard index calculation. More information on each sample can be found in Table 1.

associated coordinates, the altitude and the concentration of source salinity are shown in Table 1. Before DNA extraction, filter samples preserved in ethanol were placed in a heating chamber (70-80 °C) to evaporate the total amount of ethanol and cooled down to room temperature. DESS samples were vortexed for at least 2 min, centrifuged (4000 *g*, 4 °C, 20 min) and the supernatant was discarded. DNA extraction was performed directly within CryoTubes™ (Thermo Fisher Scientific, Waltham) using the Quick gDNA ™ Mini Prep Kit (Zymo Research Corporation, CA, USA). Genomic Lysis Buffer was applied (2100 µl) on the filters, vortexed for 2 min and centrifuged (4000 *g*, 4 °C, 15 min). Tubes were vortexed again and incubated at room temperature for 15 min. Further steps were performed applying the manufacturers' protocol except for an additional washing step. The quality of each sample as well as the amount of DNA was measured via a Nanodrop Spectrophotometer ND-1000 (Peqlab, VWR, Erlangen, Germany) and stored afterwards at -20°C.

DNA isolation and sequencing of mock community

In total, ten known strains from six various supergroups were used as an artificial sample for sequencing (mock community): Alveolata (*Protocruzia* sp. (MT355146); *Aristerostoma* sp. (MT081566)), Ancyromonadida (*Fabomonas tropica* (MT355148)), Rhizaria (*Massisteria* sp. (MT355122)), Discoba (Rhynchomonadidae sp. (MT355133), *Neobodo* sp. (MT355124)), Stramenopila (*Cafeteria burkhardae* (MN315604); Bicosoecida sp. (MT355117)) and Opisthokonta (*Enibas tolerabilis* (MH687869), *Ministeria vibrans* (MT355150)). All strains are deposited in the Heterotrophic Flagellate Collection Cologne (HFCC) and sequences were made available on NCBI Genbank. Information, like the expedition, the exact geographical location with the associated coordinates and the altitude can be found in Table S1. 18S rDNA amplification was performed by DNA extraction and standard polymerase chain reaction (PCR). Clonal cultures (30 ml) were

Table 1. Information of filtered water samples, including the exact geographical location with the associated coordinates, the altitude, the salinity, and the volume of filtered water. Which Mids were used, and the measured ionic composition for each sample are also listed here.

	Sampling Location		Filter	Lat/Long	Salinity [PSU] / Altitude [m]	Sample	Volume [ml]	Mid	Na [mmol/l]	K [mmol/l]	Ca [mmol/l]	Mg [mmol/l]	Cl [mmol/l]	S [mmol/l]	As [mmol/l]	B [mmol/l]	Ba [mmol/l]	Li [mmol/l]	P [mmol/l]	Si [mmol/l]	Sr [mmol/l]
1	Salar de Surire	S28	Filter-17_009	18°47'50.97"S 69° 4'57.05"W	50 / 4260	Lake	150	UDI_002	617.55	49.49	22.35	25.91	657.05	115.28	0.57	27.58	0	11.3	0	0	0.23
		S19	Filter-17_010	18°47'42.40"S 69° 5'7.69"W	154 / 4267	Lagoon	250	UDI_002	3413.84	220.32	15.70	303.63	969.36	1620.93	0.13	90.86	0	68.30	0.05	0.03	0.14
2	Salar de Huasco	S14	Filter-18_029	20°15'45.1"S 68°52'33.4"W	1 / 3790	Pipe	500	UDI_007	3.1	0.14	1.12	0.34	1.23	0.87	0	0.28	0	0	0	0.69	0
		S41	Filter-17_007	20°17'3"S 68°53'15"W	60 / 3765	Main Waterbody	300	UDI_007	607.26	48.01	8.42	20.9	439.06	216.5	0.23	20.85	0	6.39	0	0.58	0.08
		S31	Filter-18_025	20°17'3.6"S 68°53'15.9"W	97 / 3770	Main Waterbody	100	UDI_005	1427.54	91.50	13.41	357.56	>6126.57	596.79	0	36.81	0	0	0	0.94	1.71
		S06	Filter-18_030	20°18'18.3"S 68°53'4.4"W	294 / 3767	South Pool 2	60	UDI_008	>4388.35	>481.32	8.78	138.93	3089.36	1074.05	0.21	157.66	0	59.11	0	0.28	0.11
3	Salar de Coposa	S02	Filter-19_018	20°40'41.088"S 68°41'34.032"W	2.5 / 3729	Main Waterbody	400	UDI_003	--	--	--	--	--	--	--	--	--	--	--	--	--
		S33	Filter-19_017	20°40'41"S 68°41'36"W	30 / 3729	Pool	400	UDI_008	248.00	13.34	33.98	40.65	243.54	60.65	0	4.09	0	1.88	0	0.45	0.45
		S42	Filter-19_016	20°40'41"S 68°42'09"W	40 / 3729	Pool	400	UDI_008	431.07	18.77	61.58	53.68	574.49	54.52	0	2.9	0	3.25	0	0.31	0.31
4	Salar de Llamará	S29	Filter-18_047	21°16'13.8"S 69°37'11.1"W	18 / 743	Pond	250	UDI_003	206.14	3.64	19.2	4.94	205.92	49.30	0	0	0	0	0	1.25	0.05
		S39	Filter-17_004	21°16'13"S 69°37'11"W	41 / 740	Pool - 6	1000	UDI_004	599.02	10.25	19.2	11.49	569.02	91.92	0.03	1.76	0	0	0	1.63	0.07
		S40	Filter-18_046	21°16'4.5"S 69°37'1.3"W	133 / 726	Pool	100+200 VE	UDI_005	2278.23	39.68	20.08	32.87	2285.93	313.25	0.12	5.97	0	1.40	0	1.16	0.33
		S25	Filter-18_044	21°16'6.8"S 69°37'0.4"W	141 / 734	Main Waterbody	150+300 VE	UDI_011	2488.53	40.46	17.28	36.43	2167.45	315.34	0.15	7.28	0	1.66	0	1.26	0.37
5	Salar el Tatio	S21	Filter-18_017	22°36'35.3"S 68°03'37"W	11/3995	--	150	UDI_004	112.49	4.46	0	6.47	110.33	7.99	0.18	5.83	0.01	0	0	0.84	0
6	Salar de Atacama	S05	Filter-18_010	23°17'16.6"S 68°10'26.6"W	64 / 2301	Brine Shrimp Pool	250	UDI_007	867.20	76.02	11.02	79.85	1249.70	47.64	0.01	17.13	0	36.41	0	0.94	0.08
		S15	Filter-18_012	23°17'21.1"S 68°10'40.5"W	97 / 2301	Main Waterbody	250	UDI_008	1287.85	97.97	29.31	119.47	1669.15	92.52	0.03	26.83	0	50.33	0	0.92	0.28
		S17	Filter-18_014	23°17'22"S 68°10'42.3"W	198 / 2304	Red Pool	250	UDI_012	2610.06	204.74	25.03	244.54	3297.14	168.35	0.10	59.54	0	101.59	0	0.99	0.54
7	Salar de Laco	S03	Filter-18_023	23°51'12.1"S 67°25'34.2"W	4 / 4215	--	50	UDI_004	71.6	3.91	4.52	3.69	106.04	6.17	0	1.84	0	0.37	0	1.23	0.1
8	Salar de Talar	S34	Filter-18_019	23°55'35.9"S 67°39'40.5"W	4 / 3930	--	250	UDI_011	35.48	2.40	9.30	4.82	100.05	8.77	0	0.77	0	0.16	0	1.79	0.05
9	Laguna Tuyajto	S35	Filter-18_020	23°57'10"S 67°35'40.5"W	348 / 4019	--	35	UDI_012	>4427.89	337.58	8.09	>667.62	>5780.23	303.40	0.02	76.12	0	39.40	0	0.13	0.29
10	Salar de Pajonales	S30	Filter-18_006	25°10'10"S 68°52'24"W	264 / 3511	Sample 1	250	UDI_004	3193.34	63.98	277.7	295.72	4513.62	12.21	0.05	32.71	0.02	9.79	0	0.71	1.24
11	Salar Diego de Almagro	S11	Filter-18_004	26°15'38"S 70°06'17"W	12 / 956	Sample 1/2	250	UDI_003	85.24	4.04	22.02	2.43	121.42	23.51	0	1.59	0.01	0	0	1.08	0.12

transferred into 50 ml tubes (Sarstedt, Nümbrecht, Germany) and centrifuged (4000 *g*, 4 °C, 20 min).The supernatant was discarded, DNA was extracted using the Quick gDNA ™ Mini-Prep Kit (Zymo Research Corporation, CA, USA) applying the manufacturers' protocol, and 3 µl of the isolated DNA was used as a template together with 5 µl of each universal primer with a final concentration of 1 µM as well as a Taq DNA Polymerase Master Mix (2x) (VWR CHEMICALS, Haasrode, Belgium; final volume of 50 µl). The amplification of the 18S rDNA with the primer pairs 18S For (5′ -AACCTGGTTGATCCTGCCAGT- 3′, (Medlin et al., 1988) and 18S Rev (5′ -TGATCCTTCCGCAGGTTCACCTAC- 3′, (Medlin et al., 1988) started with the denaturation step at 96 °C for 2 minutes, then 34 cycles of 96 °C for 30 s, 55 °C for 45 s, and 72 °C for 2.5 min, then a final elongation step for 7 min at 72 °C. The PCR products were verified by gel electrophoresis using a 1% agarose gel. PCR products were purified afterwards using the Bioscience PCR Purification Kit (Bioscience, Jena, Germany) and sequenced using the 18S rDNA primer sets at GATC Biotech, Germany. Sequences were analysed using NCBI nucleotide BLAST® (Altschul et al., 1990; Wheeler and Bhagwat, 2007).

Amplification of V9 region of filter samples, library preparation and sequencing

Extracted DNA of the filter samples and each strain of the mock community were used for the amplification of the hyper-variable region V9 of the 18S rDNA gene. The isolated DNA (50ng) was used as a template together with 1.75 µl of an adapter (MID)-tagged forward and reverse primer-pair 1389F 5′-TTGTACACACCGCCC-3′ and 1510R 5′-CCTTCYGCAGGTTCACCTAC-3′ (Amaral-Zettler et al., 2009, Table S2) with a final concentration of 10 µM as well as a Taq DNA Polymerase Master Mix (2x) (VWR CHEMICALS, Haasrode, Belgium; final volume of 50 µl). PCR amplification was carried out after de Vargas et al., 2015 (98 °C for 30 sec; 25 cycles of 10 sec at 98 °C, 30 sec at 57 °C, 30 sec at 72 °C; and 72°C for 10 min) in triplicates to receive enough DNA for Illumina sequencing. If the DNA

concentration was too low, additional replicates of amplification were carried out. PCR products were verified by gel electrophoresis (1% agarose gel), pooled, and purified afterwards using the Bioscience PCR Purification Kit (Bioscience, Jena, Germany). The DNA of all 10 representatives of the mock community was pooled to one mock community sample (mock) with a final DNA concentration of 20 ng/µl. The DNA of 8 filter samples and the mock (200 ng/µl each) were pooled to form one sample for sequencing. The length of the amplicons from the pooled sample was checked by gel electrophoresis (1% agarose gel) again and handed in at the Cologne Center for Genomics (CCG) for Next Generation Sequencing (NovaSeq, paired-end 2x150 bp).

Demultiplexing and clustering into operational taxonomic units (OTUs)

The bioinformatic pipeline was used after Frédéric Mahé, (https://github.com/frederic-mahe/swarm/wiki/Fred's-metabarcoding-pipeline).

The quality and the encoding of all raw files was checked (--fastq_chars), and forward and reverse reads were merged (--fastq_mergepairs, default parameters, --fastq_allowmergestagger) with VSEARCH v2.17.0 (Rognes et al., 2016). Only assembled data that contained both adapter (MID)-tagged primers (match: 100% of the MID, 2/3 of primer length) was used for further investigations. Primers and MIDs, as well as sequences containing nonspecific nucleotides (Ns), were removed with Cutadapt v3.4 (Martin, 2011). Reads were demultiplexed, dereplicated via VSEARCH v2.17.0 (Rognes et al., 2016), and a quality file was generated. Files from the salars were pooled (22 files), dereplicated and clustered by Swarm v3.1.0 (Mahé et al., 2021) with default settings into operational taxonomic units **(**OTUs). The five mock files were not pooled but treated separately the same way. OTU representatives were checked for chimera with VSEARCH v2.17.0 (Rognes et al., 2016). V9_DeepSea database (Schoenle et al., 2021a) was updated with additional V9 sequences of protist from the Heterotrophic Flagellate Collection Cologne and

used for taxonomic assignment with VSEARCH (global pairwise alignment –iddef 1, matching columns/alignment length). Amplicons were assigned to their best hit or co-best hits in the reference database via Stampa (Mahé, 2016). Chimeric sequences and sequences with a quality value (min. expected error rate/sequence length) higher than 0.0001 were removed, as well as reads shorter than 87 bp and OTUs with a p-identity of <80%. OTUs which could not be assigned to a reference sequence, as well as Metazoa, Fungi, Streptophyta and phototrophic organisms, were removed. The OTUs which could not be assigned to a taxonomic group at the taxonomic level were grouped as "Unknown/Uncertain". All samples of the salars were on the one hand additionally filtered separately due to their own mock (reads were discarded lower than the total read number of the next OTU after the mock OTUs) and on the other hand, they were filtered in the same way as in Schoenle et al., 2021.

Data analyses

R-Studio with the R-version 4.0.2 was used to conduct statistical analysis as well as illustrate the investigated data regarding community composition (R Core Team, 2020). The V9_DeepSea reference database (Schoenle et al., 2021a) was used for taxonomic assignment. The Jaccard index was calculated to compare the community composition and the Jaccard distance to display beta-diversity using the unweighted pair-group method with arithmetic means (UPGMA) (R package: "vegan"; "vegdist" and "hclust" function, Oksanen et al., 2018). The results were illustrated in a dendrogram using "ggplot2" (Wickham, 2009) and bootstrap values of clusters were calculated with 100,000 bootstrap replicates ("fpc" package; "clusterboot" function) (Hennig, 2007). Non-metric multidimensional scaling (NMDS) was performed using the Jaccard distance and "vegan" to show the various samples and their communities due to their similarities. Venn diagrams that show the number of shared and unique OTUs between all different salinity

ranges as well as between different samples at one location were calculated via the R package VennDiagram (Chen and Boutros, 2015). Rarefaction curves and the Shannon-Wiener index were calculated to evaluate the sequencing depth (R package: "vegan"; Oksanen et al., 2018)

Chemical composition

The ion concentrations of water samples were analyzed at the University of Cologne using a Spectro Arcos inductively coupled plasma optical emission spectrometer (ICP-OES, SPECTRO Analytical Instruments, Kleve, Germany) with axial plasma observation. The Spectro Arcos is capable to simultaneously monitor line intensities at wavelengths between 130 and 770 nm using a Paschen-Runge mount equipped with 32 CCD detectors. Sample nebulization and introduction into the plasma is accomplished using a cross-flow nebulizer mounted on a Scott double pass spray chamber. The operating parameters for ICP-OES analyses can be found in Table S6 in the supplementary material. The emission intensities were monitored at the following wavelengths: Al 167.078 nm, As 189.042 nm, B 249.773 nm, Ba 455.404 nm, Ca 317.933 nm, Cl 134.724 nm, Cu 324.754 nm, Fe 259.941 nm, K 766.491 nm, Li 670.780 nm, Mg 280.270 nm, Na 589.592 nm, P 177.495 nm, S 182.034 nm, Si 251.612 nm, Sr 407.771 nm, V 292.464 nm.

Isolation Approach

All Bachelor- Master- and PhD theses from our working group (2014-2021) that worked on salar samples from the Atacama Desert were checked and listed regarding the isolation of organisms. All organisms from the different salars were listed separately and assigned to their supergroups.

Results

Mock community

Each mock community was evaluated separately. After the first filter steps (removing chimera, sequences with a quality value > 0.0001, reads < 87 bp, OTUs p-identity < 80%, Metazoa, Fungi, Streptophyta and phototrophic organisms), each mock OTU table still ended up with 1147-1393 OTUs (see Table S4). Total reads of the next OTU after the mock community representatives were used as an additional filter for the salar samples (Mock S09: 1430 reads, Mock S18: 2317 reads, Mock S27: 2513, Mock S36: 1668, Mock S45: 1512, Table S4). After this additional filter, the OTU table ended up with the OTUs of the mock community only.

High-throughput sequencing of salar samples

The sequencing of 22 salar samples resulted in 221,957,500 raw reads with both adapter-tagged primers, on average with 10.0 ± 5.8 million per sample. After removing reads that were too short, we ended up with 119,032,169 reads (80,237 OTUs), on average with 5.4 ± 3.5 million per sample. After removing chimera, sequences with a quality value > 0.0001, reads < 87 bp, OTUs p-identity < 80%, we ended up with 21,848 operational taxonomic units (OTUs) (99,241,596 reads). A 97% identity threshold resulted in 3,771 (79,052,610 reads) eukaryotic OTUs. All 22 samples showed a nearly saturated rarefaction curve with slopes in the range of 0 (S02) to $3{,}13 \times 10^{-4}$ (S34) (Table S3). The alpha diversity, calculated via the Shannon index ranged from 0.19 to 4.26, with a mean value of 1.71 (Table. S3). Removing Metazoa, Fungi, Streptophyta and phototrophic organisms (OTUs p-identity < 80%), resulted in 13,918 OTUs (41,370,053 reads) which could be assigned to heterotrophic protists (Table S5). The average amplicon size of the V9 region of 18S rDNA sequences was 125 bp (± 8 bp, standard deviation). The salar samples were additionally filtered by the mock: S02, S03, S05, S06 by Mock S09; S11, S14, S15, S17 by Mock S18; S19, S21, S25 by Mock S27; S28, S29, S30, S31, S33, S34, S35 by Mock

S36 and S39, S40, S41, S42 by Mock S45. Appling the additional filter of the mock community, the salar sample dataset ended up with 461 OTUs (39,716,694 reads) of heterotrophic protists for all salar samples (3.3%). On average, unfiltered OTUs could be assigned to reference sequences with a p-identity of 66 % (Figure S2). 16.9 % of all filtered OTUs could be assigned with 100% identity to a reference sequence from the database of which 2.6 % of OTUs were unknown or uncertain. 13.8 % of OTUs could be assigned to sequences with a similarity of 98 %. Most filtered OTUs from 80-100% similarity to reference sequences could be assigned to Alveolata (35.6 %) and Stramenopiles (27.8 %) (Figure 2, S2). Within the Stramenopiles, 2.6 % of OTUs could be assigned to Placididea.

Community composition

The calculation of the beta diversity of all 22 samples, collected from salars in the Atacama Desert, formed five separated clusters. The first cluster was represented by one freshwater sample from a Groundwater inflow from the Salar de Huasco (1 PSU, 3790 m) and a brackish water sample from the Salar el Tatio (11 PSU, 3995 m). The second cluster was formed by two freshwater samples (4 PSU) from Salar de Laco (4215 m) and Salar de Talar (3930 m) which both are located at altitudes of almost 4000 m or more meters. The third cluster contains two hyposaline samples. The pond sample from Salar de Llamará (S29, 18 PSU) and the sample from Salar Diego de Almagro (S11, 12 PSU). The fourth cluster consists of samples from the Salar de Huasco, The Salar de Llamará and Salar de Atacama. Samples from the same location cluster within this cluster also together. The fifth cluster contains all further samples from locations higher than 2000 m. Also within this clade, the samples from the Salar de Coposa cluster together. The first four clusters were supported moderately (bootstrap: 0.73-0.87), the fifth and largest cluster was not well supported (bootstrap: 0.45) (Figure 2). The samples itself are mainly dominated by Stramenopiles (53.1 % Ochrophyta and 46.9 % non-Ochrophyta),

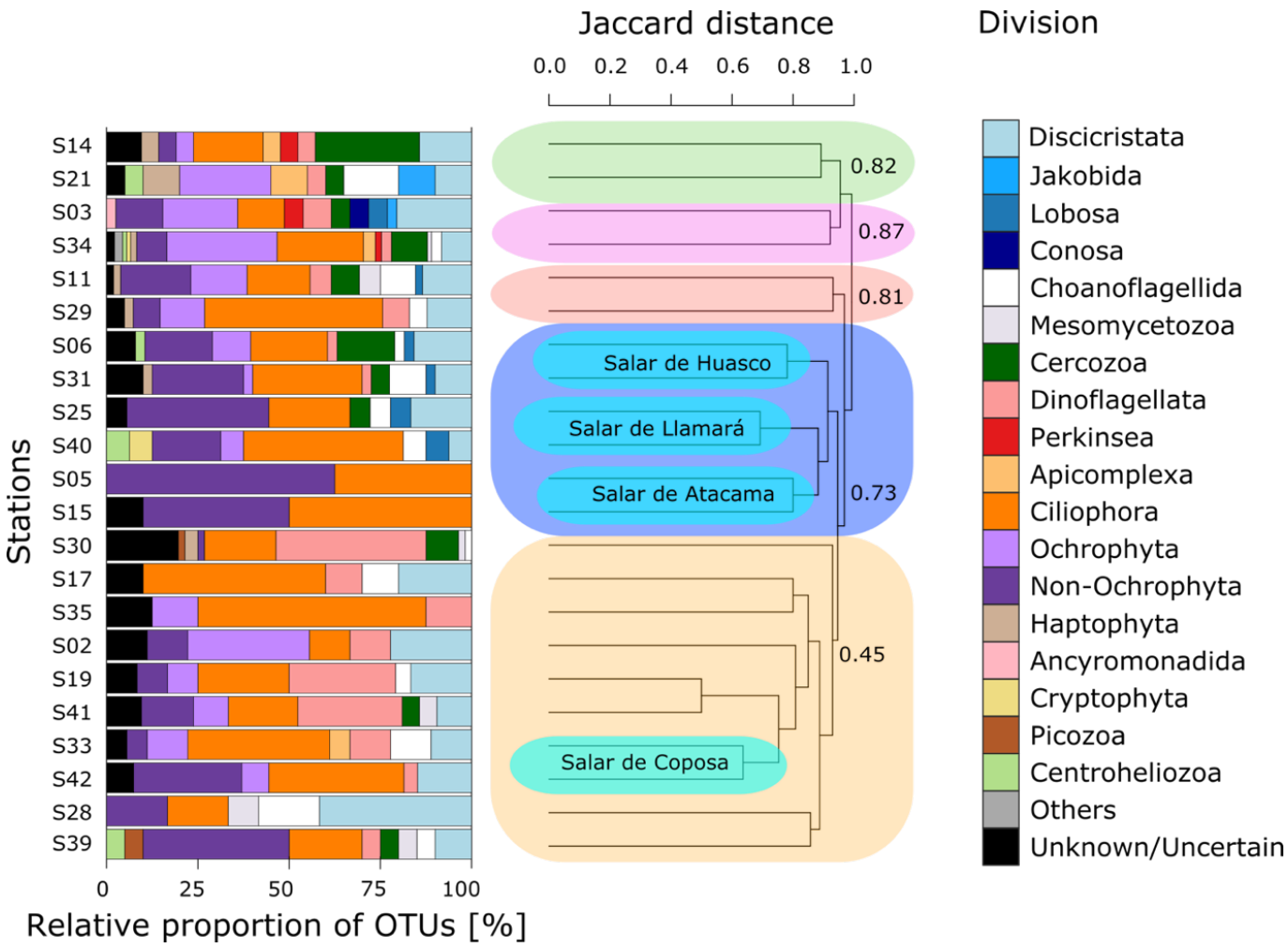

Figure 2. Community composition and clustering of inland water samples. Relative proportion of OTUs was assigned to taxonomic groups. Others - Relative proportion of OTUs <1 %; Unknwn/Uncertain - OTUs could not be assigned to a taxonomic group. Clusters of the dendrogram, based on the Jaccard index, are highlighted and bootstrap values are given for each cluster.

and Alveolata (68.3 %.Ciliophora). Ochrophyta are mainly represented by the class Chrysophyceae (88.2 %), Stramenopiles (non-Ochrophyta) by Bicosoecida (38.3 %), Placididea (20 %) and Oomycota (16.7 %). Ciliophora are mainly represented by Oligohymenophorea (31.3 %), Spirotrichea (25 %), Litostomatea (13.4 %) and Phyllopharyngea (10.7 %). In some samples Cercozoa (9.3 % of all filtered OTUs) and Dinoflagellata (7.8 of all filtered OTUs) have also a higher abundance (S14, S30) as well as Discicristata (S28, 9.5 % of all filtered OTUs, of which 40.9 % can be assigned to Heterolobosea). The fifth clade contains many samples with unknown

or uncertain OTUs. The fourth clade contains samples where non-Ochrophyta are abundant as well as Ciliophora. S34 (Salar de Talar, 4 PSU, 3930 m) shows the highest (29 %), S05 (Salar de Atacama, Brine Shrimp Pool, 64 PSU, 2301 m) and S35 (Laguna Tuyajto, 348 PSU, 4019 m) the lowest (1.7 %) diversity compared to all other sampling locations.

Similarity of heterotrophic protists' OTUs at one location regarding the salinity concentration

To investigate the community composition at one location, samples, if possible, were also taken from various positions with different salinity concentrations at one salar (Salar de Huasco, Salar de Llamará, Salar de Atacama, Salar de Coposa) and compared to each other (Figure 3, 4). Four samples were compared at the Salar de Huasco (1x subsaline, 3x hypersaline) and the Salar de Llamará (1x hyposaline, 1x mesosaline, 2x hypersaline), three different samples at Salar de Atacama (3x hypersaline) and the Salar de Coposa (1x subsaline, 2x mesosaline). At the Salar de Huasco (Figure 3A) and the Salar de Llamará (Figure 3B) there were no OTUs shared between all different salinities within one salar regarding the mock community filter. Within the Salar de Atacama (Figure 4A) there were 5 % of OTUs shared between all different salinities and the samples of the Salar de Coposa shared 14 % (Figure 4B) regarding the mock community filter. The sample with the lowest and the sample with the highest salinity shared in all salars no OTUs. At the Salar de Huasco, all different samples contained nearly the same amount of unique OTUs (about 20 %). All three hypersaline samples shared only a small amount of OTUs (2 %), but the two samples with the highest salinities, considered alone (97 PSU and 294 PSU), shared a relatively high amount of OTUs among themselves (11 %) and 1 % with the subsaline sample (1 PSU, Figure 3A). Nearly the same pattern of distribution could also be found in the deep sea sediment (Schoenle et al., 2021b), and there were also OTUs shared between the highest and

lowest salinities (Figure 3A'). At the Salar de Llamará, the distribution of unique OTUs was different. The hyposaline sample with the lowest salinity (18 PSU) contained the highest percentage of unique OTUs (45 %), followed by the mesosaline sample (41 PSU), and both hypersaline samples that contained about 10 % of unique OTUs (133 PSU = 9%; 141 PSU = 12 %). The hyposaline and the mesosaline sample shared 4 %, both hypersaline samples 10 % of OTUs (Figure 3B). In the deep sea sediment highest and lowest salinities had the highest percentage of unique OTUs (Schoenle et al., 2021b) (Figure 3B'). The samples from

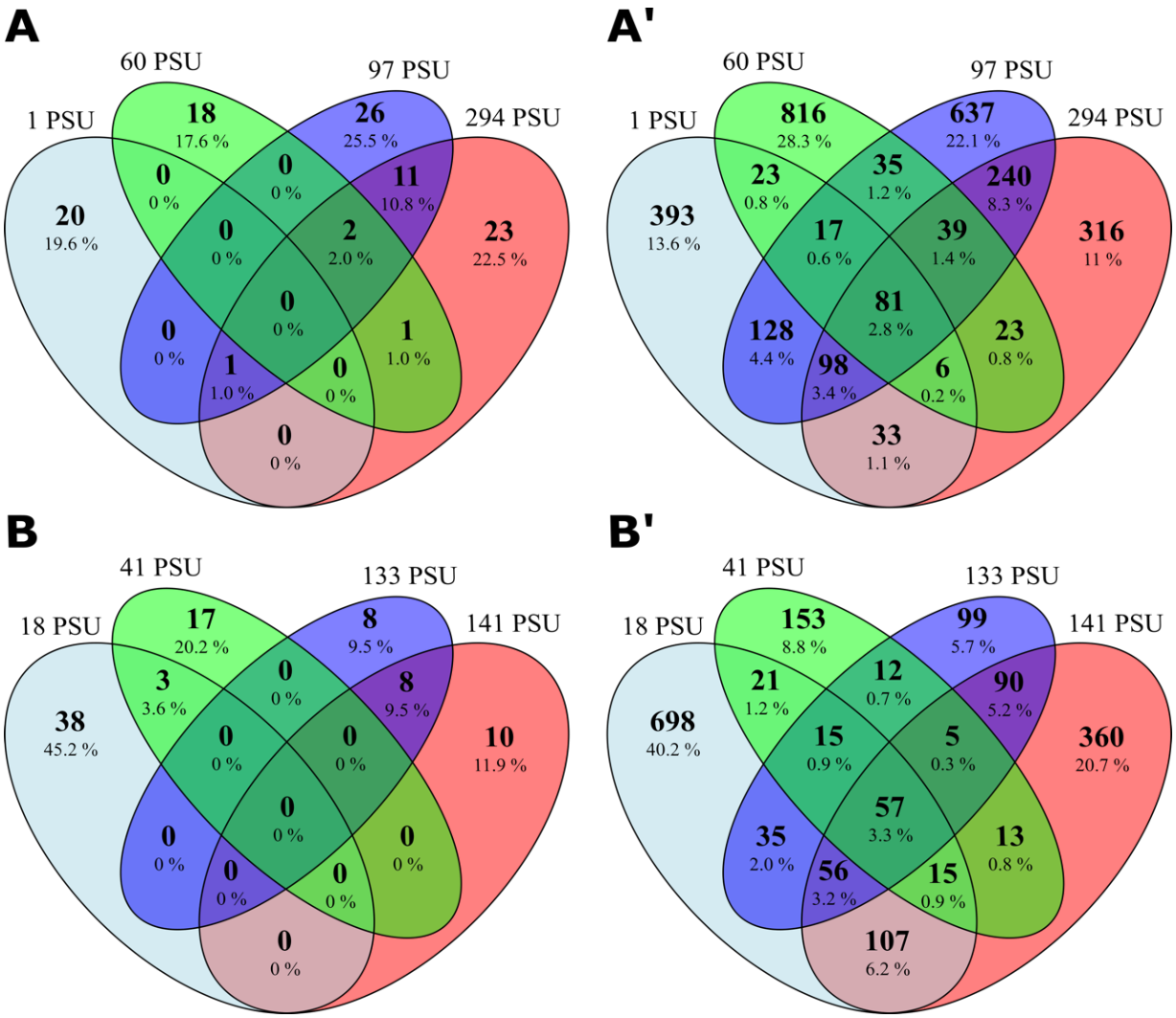

Figure 3. Comparison of heterotrophic protists' OTUs of samples from the same location. Venn diagrams show the number and percentage of unique and shared OTUs. Different salinities from one location were compared to each other. A, A' - Salar de Huasco; B, B' - Salar de Llamará. A and B were filtered regarding the mock community; A' and B' were filtered regarding Schoenle et al. (2021).

the Salar de Atacama, where three hypersaline samples were compared, had a relatively high amount of unique OTUs (23% - 32 %). The moderate hypersaline sample (97 PSU) shared 9 % of OTUs with the samples with the lowest- (64 PSU), as well as to the sample with the highest (198 PSU) salinity within this salar (Figure 4A). The other evaluation showed that all samples had a high percentage of unique OTUs. At the Salar de Coposa, the sample with the highest salinity contained the

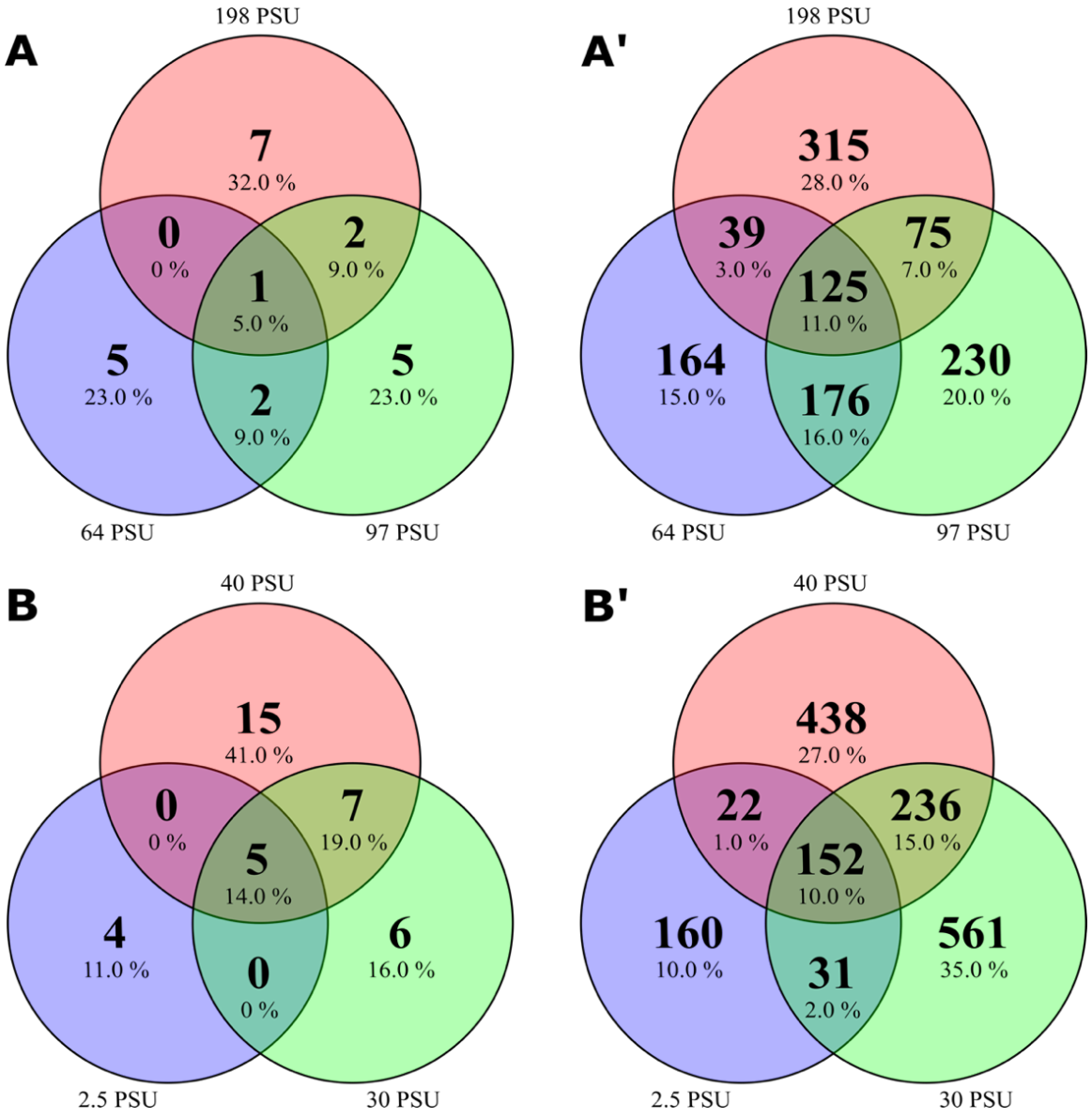

Figure 4. Comparison of heterotrophic protists' OTUs of samples from the same location. Venn diagrams show the number and percentage of unique and shared OTUs. Different salinities from one location were compared to each other. A, A' - Salar de Atacama; B, B' - Salar de Coposa. A and B were filtered regarding the mock community; A' and B' were filtered regarding Schoenle et al. (2021).

highest amount of unique OTUs and shared 19 % of OTUs to the other mesosaline sample (30 PSU) (Figure 4B). The same pattern of distribution could also be found in the deep sea sediment (Schoenle et al., 2021b) (Figure 4A',B').

Similarity of heterotrophic protists' OTUs between locations regarding the salinity concentration

The comparison of samples from different locations from subsaline to slightly hyposaline conditions showed that no OTUs were shared between all of them (Figure 5A) or only a small percentage of 2 % (Figure 5A') and most OTUs were unique to the different salars. Both subsaline samples shared either no or only one OUT (Figure 5A, A'), but the hyposaline samples shared 6 % or even 9 % of OTUs. But also the lowest subsaline and one hyposaline samples shared 3% of OTUs (Figure 5A, A'). The comparison of samples from different locations from hyposaline to slightly mesosaline conditions showed also that no OTUs were shared between all of them (Figure 5B) or only a small percentage of 3 % (Figure 5B') and most OTUs were unique to the different salars. All three hyposaline samples shared no or only a low percentage (0.3 %) of OTUs. The highest hyposaline sample shared 4 or 6 % of OTUs with the mesosaline sample, the lowest hyposaline sample shared no or only a small amount (2 %) of OTUs with the mesosaline sample (Figure 5B, B'). The comparison of samples from different locations with approximately the same salinity showed that no OTUs were shared between all of them and most OTUs were unique to the different salars (Figure 6A). The sample from the Salar de Coposa contained the highest- (22 %) the sample of the Salar de Atacama the lowest (6 %) amount of unique OTUs. The sample from the Salar de Coposa shared 5 % of OTUs with the Salar de Huasco as well as with the Salar de Atacama. But the Salar de Atacama has only 2 % with Salar de Huasco and Salar de Coposa in common. All other comparisons of the different locations showed that either no OTUs were shared or at most 2 – 5 %. The filtering approach

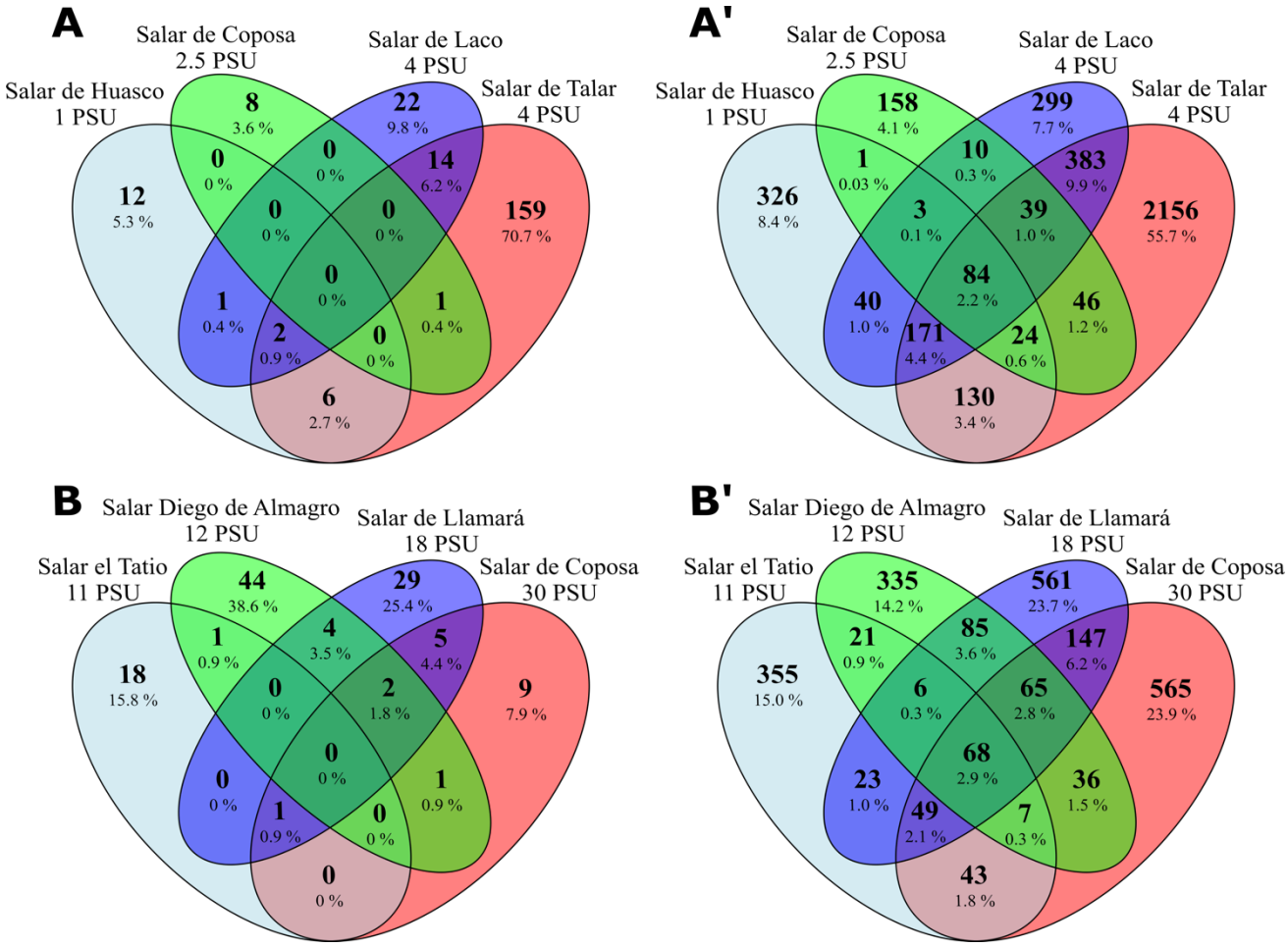

Figure 5. Comparison of heterotrophic protists' OTUs of samples from different locations with A – subsaline to slightly hyposaline and B – hyposaline to slightly mesosaline water conditions. Venn diagrams show the number and percentage of unique and shared OTUs. A and B were filtered regarding the mock community; A' and B' were filtered regarding Schoenle et al. (2021). A – Salar de Huasco (S14), Salar de Coposa (S02), Salar de Laco (S03), Salar de Talar (S34 - 4 PSU); B' – Salar el Tatio (S21), Salar Diego de Almagro (S11), Salar de

after Schoelne et al. (2021b) showed that the Salar de Huasco had the most unique OTUs (592 OTUs, 28.8 %) and the Salar de Surire the lowest (77 OTUs, 3.4 %). All samples had 2 % of OTUs in common (Figure 6A'). The comparison of the highest hypersaline samples from different locations showed only a small amount of OTUs was shared between all of them (1 % - Figure 7A, 2 % Figure 7A') or a small percentage and most OTUs were unique to the different salars. The sample from the Salar de Pajonales contained the highest- (both filter methods, 38 % or 41%) amount of unique OTUs, the samples from the Laguna Tuyajto (both filter methods, 3 % or 4 %) and the Salar de Atacama the lowest (both filter methods, 5 % or 2 %).

Salar de Pajonales shared the most OTUs with Salar de Surire (both filter methods, 9% or 13 %), but all other comparisons of the different locations showed that nearly 0 to 2 % were shared (Figure 7A). The salinity was related to the OTU composition of all samples within a NMDS plot (Figure 8). The NMDS plot showed that all hypersaline samples and also most of the hyposaline samples cluster together regarding their OTU composition. Two samples from the Salar de Coposa (mesosaline samples) cluster together with the hypersaline samples. The other mesosaline samples cluster together with another subsaline sample from the Salar de Coposa. At least two samples from one Salar cluster in the immediate vicinity (Salar de Llamará, Salar de Atacama, Salar de Huasco, Salar de Coposa). But not all samples from one location cluster together, there is one sample that do not cluster with the other ones from this location (Salar de Huasco, the Salar de Surire and Salar de Huasco) (Figure 8).

Chemical composition

To relate the metabarcoding data to the habitat, we also investigated the concentration of different ions (Figure 9). In all different salar samples, the dominant ions were sodium (mean = 31 %) and chlorine (mean = 44 %). The highest proportion of sodium occurred in a sample from the Salar de Surire (S19, 51 %), of chlorine in a sample of the Salar de Huasco (S31, 71 %). Silicon (Si) occurred in one sample from the Salar de Huasco (S14) in high concentrations (9 %). Sulfur occurred at an average of 7 % in all samples, the highest amount occurred in the Salar de Surire (S19, 24 %). Calcium and magnesium on average occurred in 3 %, potassium in 2 %. The highest values of calcium occurred in the Salar de Huasco (S14, 14 %) and the highest values of magnesium occurred in samples from the Laguna Tuyajto and the Salar de Coposa (Magnesium, S35 and S33, 6 %). The highest potassium value occurred at the Salar de Huasco (S06, 5 %). Samples of the Salar de Atacama showed also relatively high lithium values (S17, S05, 2%).

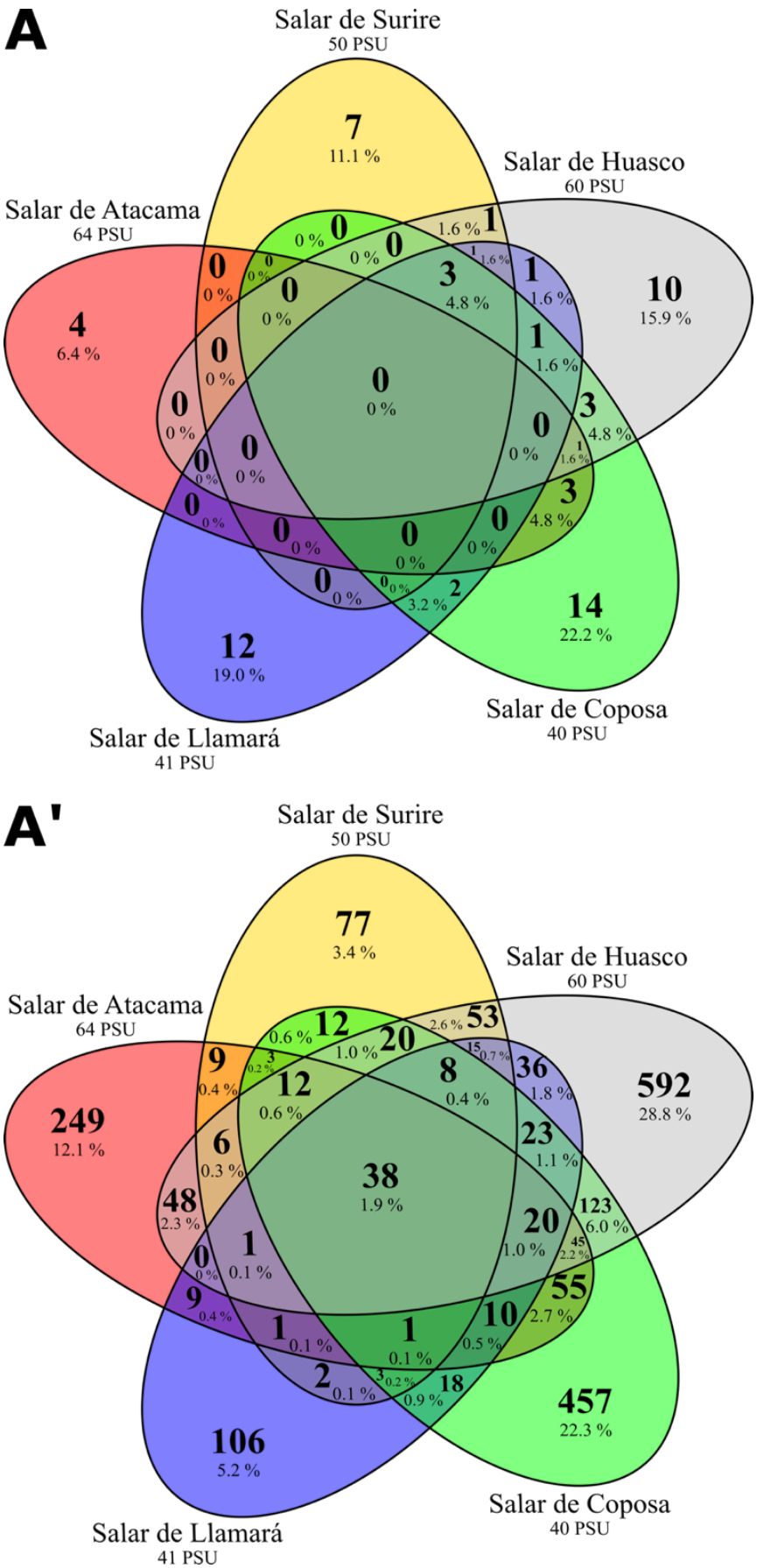

Figure 6. Comparison of heterotrophic protists' OTUs of samples from different locations with nearly the same salinity. Venn diagrams show the number and percentage of unique and shared OTUs. A were filtered regarding the mock community; A' were filtered regarding Schoenle et al. (2021).

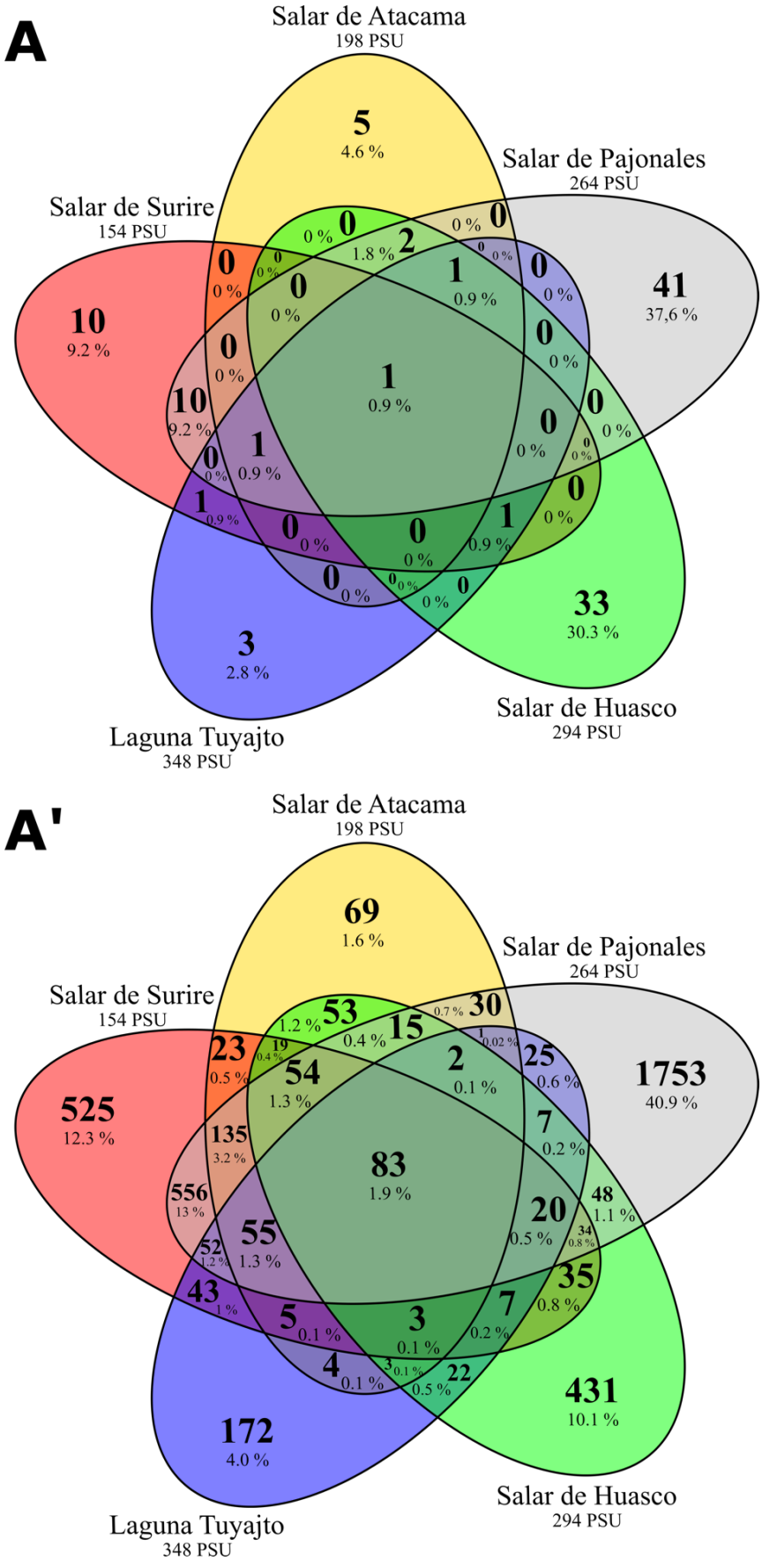

Figure 7. Comparison of heterotrophic protists' OTUs of samples from different locations with extreme high hypersaline conditions. Venn diagrams show the number and percentage of unique and shared OTUs. A were filtered regarding the mock community; A' were filtered regarding Schoenle et al. (2021).

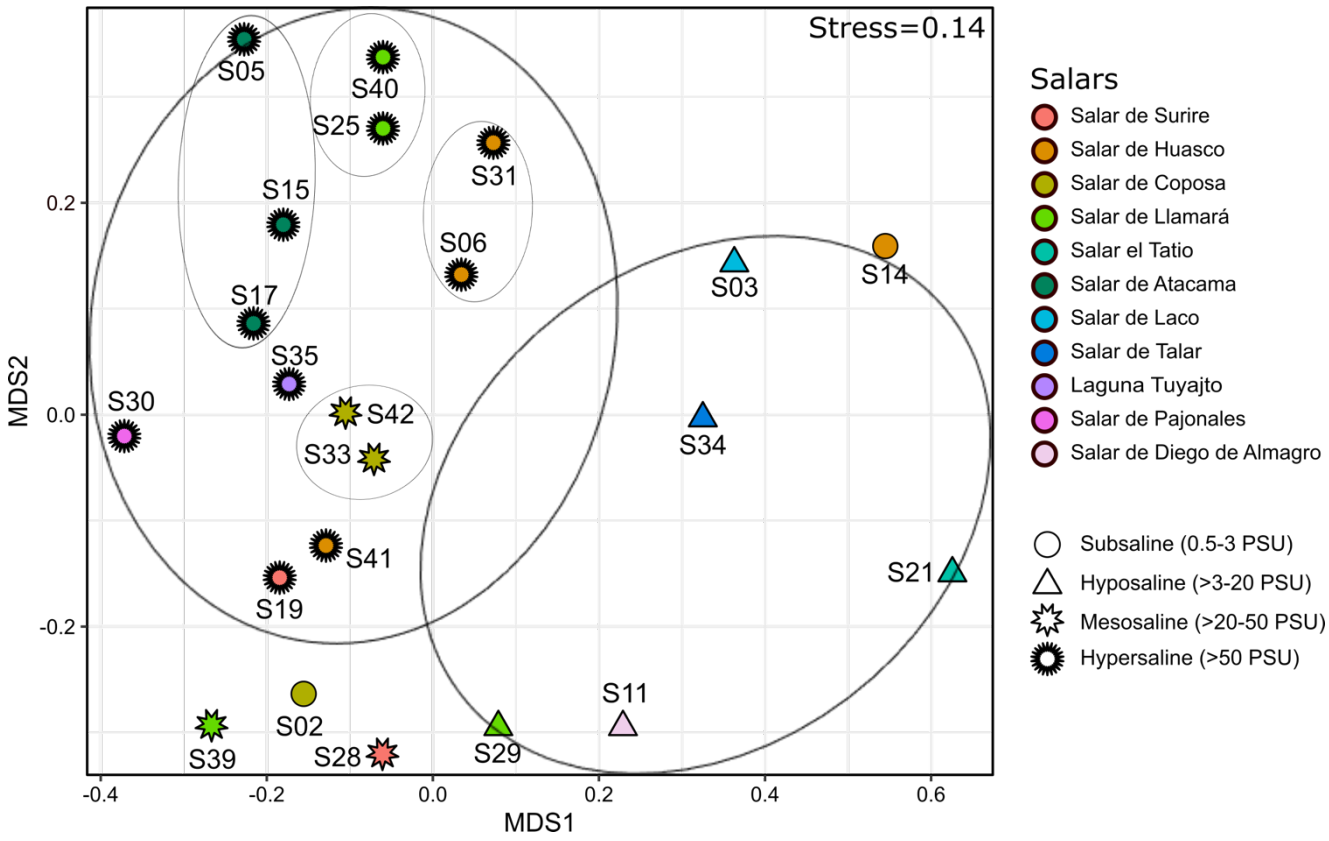

Figure 8. Non-metric multidimensional scaling (NMDS) plot based on the Jaccard Index. Different symbols indicate the classification regarding salinity concentration. Circles indicate a possible grouping regarding the community composition and the salinity.

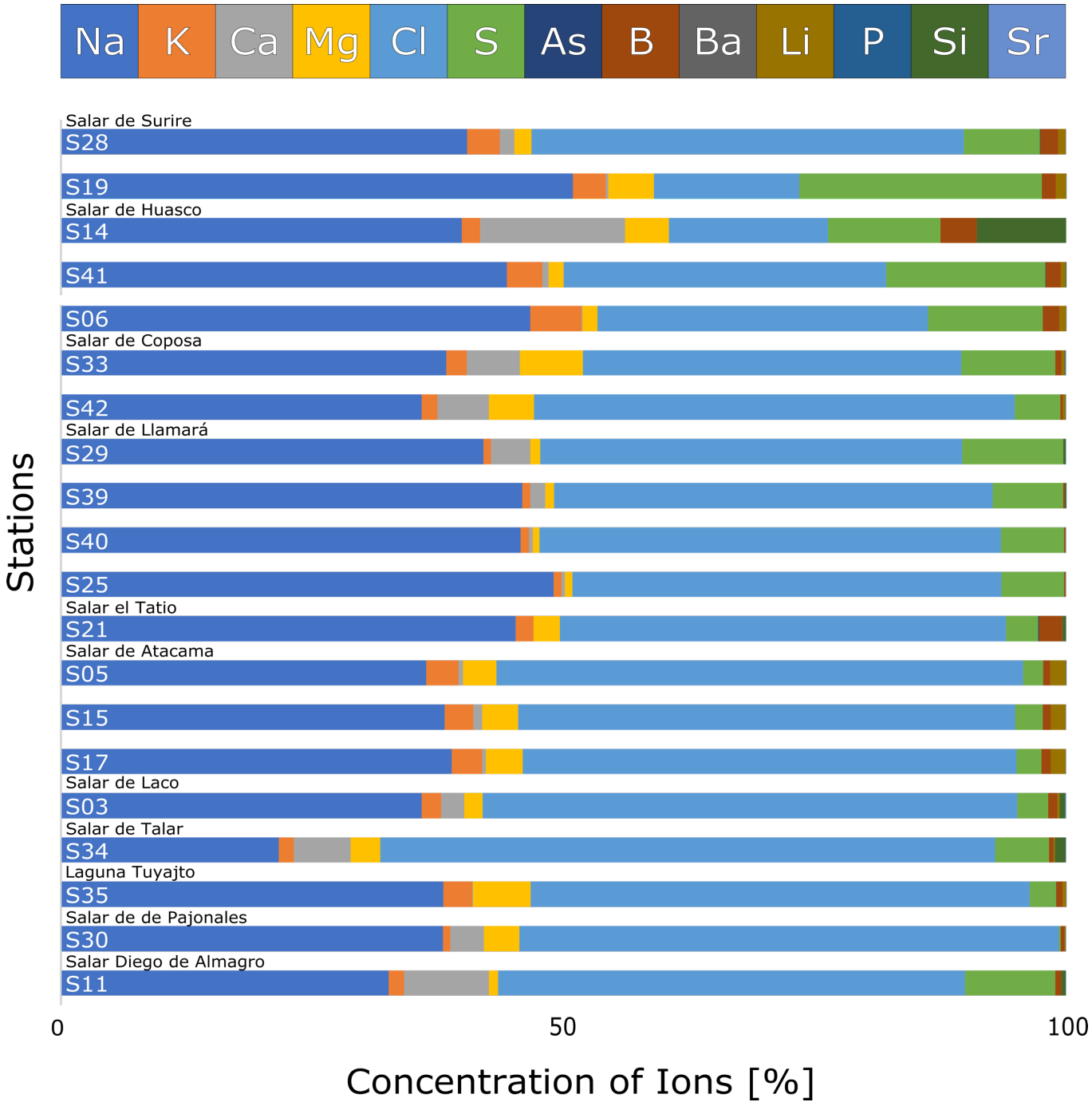

Figure 9. Relative proportion of the ionic composition investigated via the Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) for most salar samples. Exact concentrations of ions can be found in Table 1.

Isolation Approach

Additionally, we checked which further organisms were isolated in our lab from the samples we took in the Atacama Desert (Figure 10). In eight of in this study investigated salars (Salar de Surire, Salar de Huasco, Salar de Coposa, Salar de Llamará, Salar de Atacama, Salar de Laco, Salar de Talar and Salar Diego de Almagro) there were further representatives isolated of various supergroups. Stramenopiles (Chrysophyceae, Bicosoecida, Placididea) occurred in six salars, except in the Salar de Laco and Salar Diego de Almagro (Figure 10). Discoba (Heterolobosea) and Obazoa (Apusomonadida, Choanoflagellatea) were isolated from five salars, Rhizaria (Cercozoa) and Alveolata (Ciliophora) from two salars. Cryptista (Cryptophyta) were the only representatives that were only isolated from one salar (Salar de Coposa) up to now.

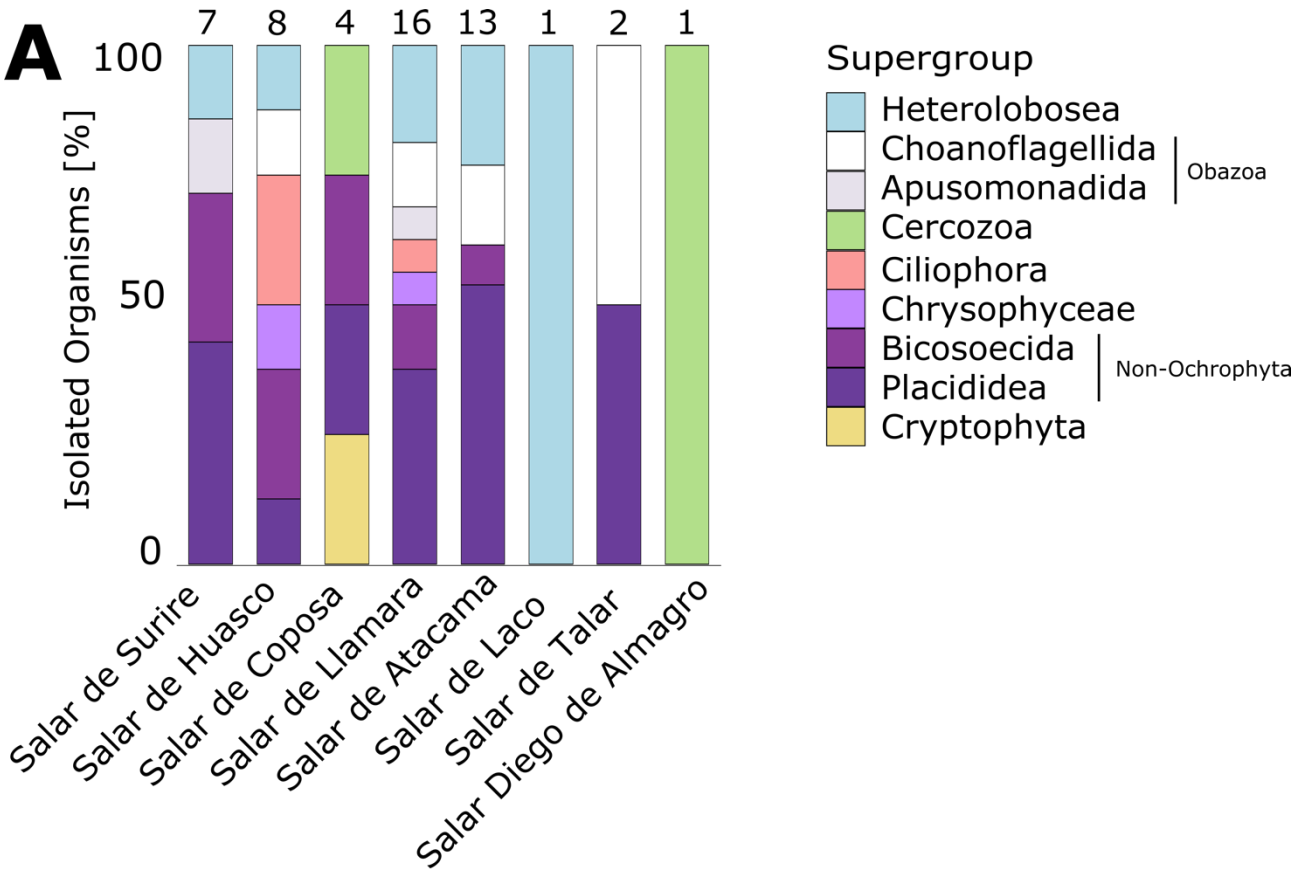

Figure 10. Relative abundance of isolated, cultivated, and sequenced strains from various salars in the Atacama Desert. Numbers above the bar indicate the total number of isolated strains from this location.

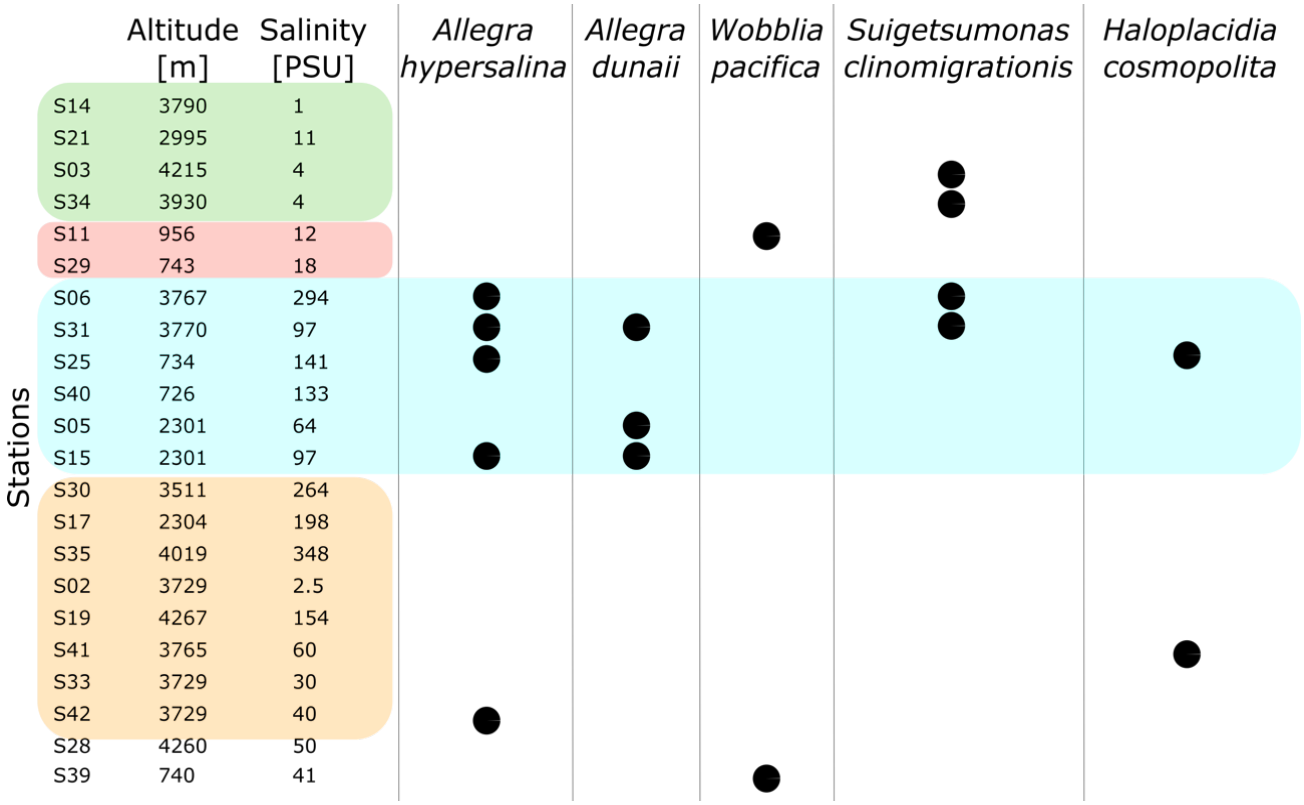

Figure 11. Genotype identification of Placididea within the different samples. The mock community filtered data was used for this identification. Black dots indicate the presence of this genotype in the sample with 100 % identity. Coloured clusters indicate the clusters that were also obtained by the Jaccard clustering.

In the next step, we checked the presence of OTUs belonging to the class of Placididea in all samples (Figure 11). Only OTUs with an identity of 100 % to a known placicid species were included. Primarily, OTUs of placidids could be found in brine water. The genotype of *Allegra hypersalina* was detected in four different salars, mainly in brine water (Salar de Huasco (S06, 297 PSU; S31, 97 PSU), the Salar de Llamará (S25, 141 PSU), the Salar de Atacama (S15, 97 PSU)) but also in saline water (Salar de Coposa; S42, 40 PSU). The genotype of *Allegra dunaii* was only found in brine water samples, the Salar de Huasco (S31, 97 PSU) and the Salar de Atacama (S05, 64 PSU; S15, 97 PSU). The genotype of *Haloplacidia cosmopolita* was detected at two brine water locations: at the Salar de Huasco (S41, 60 PSU) and the Salar de Llamará (S25, 141 PSU). The genotype of *Wobblia pacifica* was localized

within brackish- (Salar Diego de Almagro, S11, 12 PSU) and saline waters (Salar de Llamará, S39, 41 PSU). The genotype of *Suigetsumonas clinomigrationis* was found in freshwater- (Salar de Laco, S03, Salar de Talar, S34; both 4 PSU) and saline water samples (Salar de Huasco, S06, 297 PSU; S31, 97 PSU).

Discussion

We investigated 22 different samples from 11 inland waters in the Atacama Desert (Chile) to get an idea regarding the overlap or separation of the unicellular eukaryote communities. The V9 region of 18S rDNA turned out to be a good tool to understand the diversity of the microbial community if the read number is taken into account (Choi and Park, 2020). Within our metabarcoding approach, we conducted two different filter methods and compared them to each other. As previous studies showed, Stramenopiles, (Ochrophyta and non-Ochrophyta but also Alveolata (Ciliophora) were isolated from hypersaline environments (Park and Simpson, 2010; Qu et al., 2020; Rybarski et al., 2021; Schoenle et al., in prep, Chapter 1,3). All samples from our metabarcoding approach were also mainly dominated by representatives of these groups. This initially suggested that the general species composition also could be the same at different hypersaline salars. However, comparing the samples at within one salar, it was noticed, that all samples contained a possibly different community structure, and only a tiny part of OTUs overlapped. It could also be seen that the samples that showed a similar salinity shared more OTUs than those that differed greatly in their salinity. This could be observed in all salars from which several samples of different salinity were taken (Salar de Huasco, Salar de Llamará (Figure 4); Salar de Atacama, Salar de Coposa (Figure 5)). A similar pattern emerged comparing the samples from different inland waters regarding their salinity. Samples from the same salinity ranges (subsaline, hyposaline, mesosaline, hypersaline) showed only a slight overlap of the communities and not only each separated area with a different

salinity at one salar is unique and diverse, but also the different salars show all a unique community. Investigations of unique sequences from different places regarding different salinities showed that most unique sequences could also be assigned to Stramenopiles and Alveolata. This suggests that the salars can be considered as fairly separated systems with only a small exchange and where evolution could be observed. The organisms seem to be restricted between different salars, but also within one salar. Regarding the Jaccard index, all hypersaline communities can be clearly differentiated from the hyposaline samples and do not cluster with these, but together with all other hypersaline samples (Figure 8). These different environments seem to be very diverse, and the organisms might be particularly adapted to these conditions. The water composition of the different samples of the salars is very similar, with sodium and chlorine as the dominant ions. Furthermore, most samples are also rich in potassium, calcium and magnesium, and some are high in sulphur. At the Salar de Atacama were mainly hypersaline samples collected and compared. Thus, the ionic composition within each sample is very similar the community composition of each microhabitat is very diverse and overlaps only a little. At places where samples were taken from different salinity ranges, it is possible to get a slight impression of what could happen during evaporation and to which situations the organisms would have to adapt. The Salar de Llamará, for example, has a continuous inflow of groundwater. In addition to the high evaporation rate, there is no precipitation, and therefore, it stays hydrologically constant. Calcium influx from the surrounding rock can precipitate with an increased evaporation rate and is reduced in the water. At the Salar de Surire, the concentration of sulphur in the water rises with an increased evaporation rate, suggesting that it is not sulphate but calcium chloride that precipitates. These changes in the water composition require a special adaptation of the organisms which can also be seen in their unique community composition. The isolation approach was also included to understand

better which organisms can live in these places and how they are distributed. Stramenopiles were also, in this approach, the dominant representatives. Placidids, e.g. could be isolated from almost all salars, and through previous studies from (Rybarski et al., 2021), it was also possible to compare the V9 region of 18S rDNA within this group (Table S7). There are currently only two species that contain the same V9 region. The others show a relatively high variability between the species in this short V9 sequence (up to 21.5 %). Thus, it seems that placidids could possibly be distinguished down to their genera, based on the V9 region of 18S rDNA. The metabarcoding data was examined again for placidids (identity of 100%) showing that placidids occur mainly in hypersaline environments. *Allegra hypersalina* could primarily only be isolated from the Salar de Llamará so far. However, a high read number was also found in the Salar de Huasco, Salar de Atacama and Salar de Coposa. This suggests that representatives of this species may also potentially inhabit the other salars. *Allegra dunaii* could only be detected in three salars, but representatives of this species were also previously isolated from the Salar de Talar, Salar de Surire and Salar de Coposa. The representatives from the genus *Allegra* showed to be able to cope with extreme high salinity ranges (20-284 PSU), which suggests that *Allegra* is particularly well adapted to extremely changing salinities. The occurrence of species in the salars that were originally only isolated from brackish water systems (*Suigetsumonas clinomigrationis*), should also be viewed with caution and only in combination with an isolation approach. Especially in the Salar de Huasco, with its several different groundwater inflows with different compositions and salinity, it could be possible that representatives of these species could only be found in the brackish parts of the salar and form resting stages due to rising salinity. The occurrence of these organisms would still be displayed due to the DNA occurrence. Our strong but also clear filtering due to the mock community where we reduce the overestimation of community composition, would make be possible that the

information about rare organisms could be lost, so we also recommend that an isolation approach should always be included. In placidids e.g., we find a lot more sequences before the filtering. It is possible, that the salars show an even higher abundance of possible new representatives of placidids but also of other classes. In summary, the different and separated salars and protists are suitable for observing the evolution on a small scale. Due to the rare but existing exchange, it is possible to observe the adaptations of the various organisms due to their high reproduction rate, the good ability to adapt to extreme conditions, and cysts' formation. In addition, it can also be seen that the diversification may have taken place starting from the Salar de Atacama. As already described in (Arndt et al., 2020) concerning the placidids, there may have been several phases of dissemination and adaptation that we can also recognize in our study based on the similar community composition.

Author contributions

Sampling in the Atacama Region was carried out by A.E.R., F.N., and H.A.; DNA isolation was carried out by A.E.R.; A.E.R. and A.S. performed bioinformatical analyses of the data. A.E.R., C.V. and M.S. performed investigations of chemical parameters. The project was launched and supervised by H.A.; A.E.R. and H.A. wrote the manuscript. All authors reviewed and revised the manuscript.

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B02,), AR288/23, MerMet 17-97, MerMet 17-11), and to M.S. (project numbers 268236062 (SFB 1211; D….).

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Supplementary Material

High diversity and isolated distribution of aquatic protists in salars of the Atacama Desert at extremely high salinities

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Table legends

Table S1. Strains which were used as the mock community with parameters like the HFCC number, name of expedition, geographical location (lat/long), the altitude, the origin of isolation and the accession number.

Species	HFCC	Expedition	Geographic Location	Lat/Long	Altitude [m]	with/from	Accession No.
ALVEOLATA							
Protocruzia sp.	766	M139	**North Atlantic Ocean** East Vema FZ, Station S8	11°13.548'N 04°46.559'W	0	*Sargassum*	MT355146
Aristerostoma sp.	744	M139	**North Atlantic Ocean** South North, Station 953	23°33.23'N 48°05.04'W	-4282	MUC Sed	MT081566
ANCYROMONADIDA							
Fabomonas tropica	175	SO237	**North Atlantic Ocean** West Vema FZ, Station 9-3	11°41.370'N 47°57.360'W	0	*Sargassum*	MT355148
RHIZARIA							
Massisteria sp.	176	SO237	**North Atlantic Ocean** East Vema FZ, Station 4-3	10°25.110'N 31°04.610'W	-5771	Sediment	MT355122
DISCOBA							
Rhynchomonadidae sp.	171	SO237	**North Atlantic Ocean** Puerto Rico Trench, Station 12-8	19°46.010'N 66°49.990'W	-8340	MUC OW	MT355133
Neobodo sp.	828	SO233T	**Pacific Ocean** Fiji Basin, Station 17019-2	15°13.000'S 173°31.100'E	-2776	MUC Sed	MT355124
STRAMENOPILA							
Cafeteria burkhardae	203	SO237	**North Atlantic Ocean** East Vema FZ, Station 4-3	10°25.110'N 31°04.610'W	-5771	MUC OW	MN315604
Bicosoecida	768	M139	**North Atlantic Ocean** East Vema FZ, Station S8	11°13.548'N 34°46.559'W	0	*Sargassum*	MT355117
OPISTHOKONTA							
Enibas tolerabilis	1243	2017	**Chile** Salar de Ascotán	21°30′13.8″S 68°18′36.3″W	3750	--	MH687869
Ministeria vibrans	178	SO237	**North Atlantic Ocean** East Vema FZ, Station 4-3	10°25.110'N 31°04.610'W	0	Plankton net	MT355150

Table S2. Illumina adapter sequences together with the forward and the reverse primer that were used in this study. Adapter sequence is shown in bold letters.

Index name	Illumina adapter sequences + 1389F primer	Illumina adapter sequences + 1510R primer
UDI_002	5'-**GGTTATAA**TTGTACACACCGCCC-3'	5'-**TTATAACC**CCTTCYGCAGGTTCACCTAC-3'
UDI_003	5'-**CCAAGTCC**TTGTACACACCGCCC-3'	5'-**GGACTTGG**CCTTCYGCAGGTTCACCTAC-3'
UDI_004	5'-**TTGGACTT**TTGTACACACCGCCC-3'	5'-**AAGTCCAA**CCTTCYGCAGGTTCACCTAC-3'
UDI_005	5'-**CAGTGGAT**TTGTACACACCGCCC-3'	5'-**ATCCACTG**CCTTCYGCAGGTTCACCTAC-3'
UDI_007	5'-**CTAGCTTG**TTGTACACACCGCCC-3'	5'-**CAAGCTAG**CCTTCYGCAGGTTCACCTAC-3'
UDI_008	5'-**TCGATCCA**TTGTACACACCGCCC-3'	5'-**TGGATCGA**CCTTCYGCAGGTTCACCTAC-3'
UDI_011	5'-**AGTAGAGA**TTGTACACACCGCCC-3'	5'-**TCTCTACT**CCTTCYGCAGGTTCACCTAC-3'
UDI_012	5'-**GACGAGAG**TTGTACACACCGCCC-3'	5'-**CTCTCGTC**CCTTCYGCAGGTTCACCTAC-3'
UDI_014	5'-**GAGTCCAA**TTGTACACACCGCCC-3'	5'-**TTGGACTC**CCTTCYGCAGGTTCACCTAC-3'

Table S3. Slopes of rarefaction curve and Shannon index calculated for all samples, as well as the mean, the maximal and the minimal value.

	slope	**Shannon index**
S02	0	1.81
S03	1,34E-04	0.54
S05	1,30E-04	0.52
S06	1,54E-04	1.6
S11	6,75E-05	1.6
S14	9,13E-05	2.42
S15	1,26E-04	0.91
S17	1,52E-04	0.54
S19	9,19E-05	4.26
S21	1,21E-04	0.45
S25	1,62E-04	1.52
S28	4,49E-05	1.89
S29	1,97E-04	2.24
S30	2,55E-04	3.63
S31	1,83E-04	1.96
S33	2,01E-04	1.35
S34	3,13E-04	2.63
S35	7,86E-05	2.73
S39	3,83E-05	1.16
S40	1,09E-04	0.19
S41	8,75E-05	2.5
S42	1,98E-04	1.17
min	0	0.19
max	3,13E-04	4.26
mean	1,33E-04	1.71

Table S4. Evaluation of the mock community and establishment of the mock community read filter. #OTUs Total - Total number of OTUs before filtering. #OTUs After 1. filter – Chimera were removed, as well as sequences with a quality value > 0.0001, reads < 87 bp, OTUs p-identity < 80%, we ended up with almost 1000 eukaryotic operational taxonomic units (OTUs) for each mock community. Read abundance after mock was checked, and taken as additional filter (Next OTU total #reads)

Mock	#OTUs Total	#OTUs After 1. filer	Next OTU total #reads
S09	3764	1147	1430
S18	4428	1340	2317
S27	4630	1393	2513
S36	4055	1244	1668
S45	4444	1360	1512

Table S5. Filter steps of the OTUs (A) and reads (B). Chimera were removed, as well as sequences with a quality value > 0.0001, reads < 87 bp, OTUs p-identity < 80% from the OTU table, we ended up with 13,918 heterotrophic protists' operational taxonomic units (OTUs). A - Number of OTUs together with OTU loss; B - Number of reads together with reads loss. Heterotrophic protists were then filtered due to the mock community resulting in 461 OTUs (39,716,694 reads) and according Schoenle et al. (2021) (3 reads filter) resulting in 9,671 OTUs (41,365,256 reads).

A

Filter	Kept	NumOTUS	Proportion of total OTUs [%]	OTUs lost	OTUs lost [%]
None	**Total**	80237	100	0	0
NoHit	**Only Eukaryota**	66388	82.74	-13849	17.26
Chimeras	**Without Chimeras**	48693	60.69	-17695	22.05
Length	**larger 87 bp**	47989	59.81	-704	0.88
Quality	**smaller 0.0001**	33912	42.26	-14077	17.54
p_idenity	**80-100%**	21848	27.23	-12064	15.04
Exclude Fungi		20082	25.03	-1766	2.2
Exclude Metazoa		18372	22.9	-1710	2.13
Exclude Streptophyta		17995	22.43	-377	0.47
Phototrophic Protists	**Heterotrophic Protists**	13918	17.35	-4077	5.08
Mock reads filter	**Heterotrophic Protists**	461	3.31	-13457	96.69
3 reads filter	**Heterotrophic Protists**	9671	69.49	-4247	30.51

B

Filter	Kept	NumReads	Proportion of total reads [%]	reads lost	reads lost [%]
None	**Total**	119032169	100	0	0
NoHit	**Only Eukaryota**	115923629	97.39	-3108540	2.61
Chimeras	**Without Chimeras**	115754698	97.25	-168931	0.14
Length	**larger 87 bp**	115750258	97.24	-4440	0.004
Quality	**smaller 0.0001**	114721037	96.38	-1029221	0.86
p_idenity	**80-100%**	99241596	83.37	-15479441	13.00
Exclude Fungi		98248221	82.54	-993375	0.83
Exclude Metazoa		69994872	58.8	-28253349	23.74
Exclude Streptophyta		69377823	58.28	-617049	0.52
Phototrophic Protists	**Heterotrophic Protists**	41370053	34.76	-28007770	23.53
Mock reads filter	**Heterotrophic Protists**	39716694	96	-1653359	4.0
3 reads filter	**Heterotrophic Protists**	41365256	99.99	-4797	0.01

Table S6. Operating parameters for ICP-OES analyses.

Parameter	Setting
RF power	1400 W
Pump speed	30 rpm
Coolant gas flow	14.5 l·min-1
Auxiliary gas flow	1.3 l·min-1
Nebulizer gas flow	0.7 l·min-1
Torch	Quartz for axial view
Sample uptake	0.2 ml·min-1

Table S7: Pairwise identity of V9 region of the 18 S rDNA of Placididea. Type strains of species are highlighted with bold letters.

			1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	***Placilonga atlantica***	**HFCC 1505**														
2	***Placidia cafeteriopsis***	NIES-1013	16.7													
3	***Placidia abyssalis***	**HFCC 745** HFCC 739	16.7	0												
4	***Wobblia lunata***	HFCC 1405	18.8	19.5	19.5											
5	***Wobblia pacifica***	**HFCC 834** HFCC 5000	18.8	21.2	21.2	9.2										
6		AY520454	16.7	18.8	18.8	3.1	7.0									
7	***Allegra dunaii***	**HFCC 1261** HFCC 1252 HFCC 1258 GU170208 - ME8 HFCC 5009 HFCC 1221	18.0	16.4	16.4	13.6	15.8	13.7								
8	***Allegra atacamiensis***	**HFCC 5011**	18.7	21.5	21.5	16.2	17.2	15.7	13.4							
9	***Allegra hypersalina***	HFCC 5021 **HFCC 1413**	20.1	20.3	20.3	15.4	15.6	14.8	11.9	4.5						
10		JX296580	13.6	15.9	15.9	15.0	16.5	12.8	13.4	14.9	15.3					
11	***Suigetsumonas clinomigrationis***	NIES-3647	15.7	16.5	16.5	14.2	15.2	12.7	12.8	15.7	16.3	3.8				
12	***Suigetsumonas keniensis***	**HFCC 1412**	16.3	17.3	17.3	14.2	15.0	12.7	12.8	15.7	16.3	3.8	1.5			
13		KX465214	16.3	18.0	18.0	13.4	14.3	11.9	13.5	16.4	15.4	4.6	2.3	0.8		
14	***Haloplacidia cosmopolita***	HFCC 207 HFCC 1408 GU170207 - ME5 GU170212 - MESS13 **HFCC 1211** HFCC 1407 HFCC 1409 GU170213 - MESS14	13.6	17.9	17.9	12.9	15.3	14.4	14.3	14.1	14.9	15.0	17.3	15.7	14.9	

Figure legends

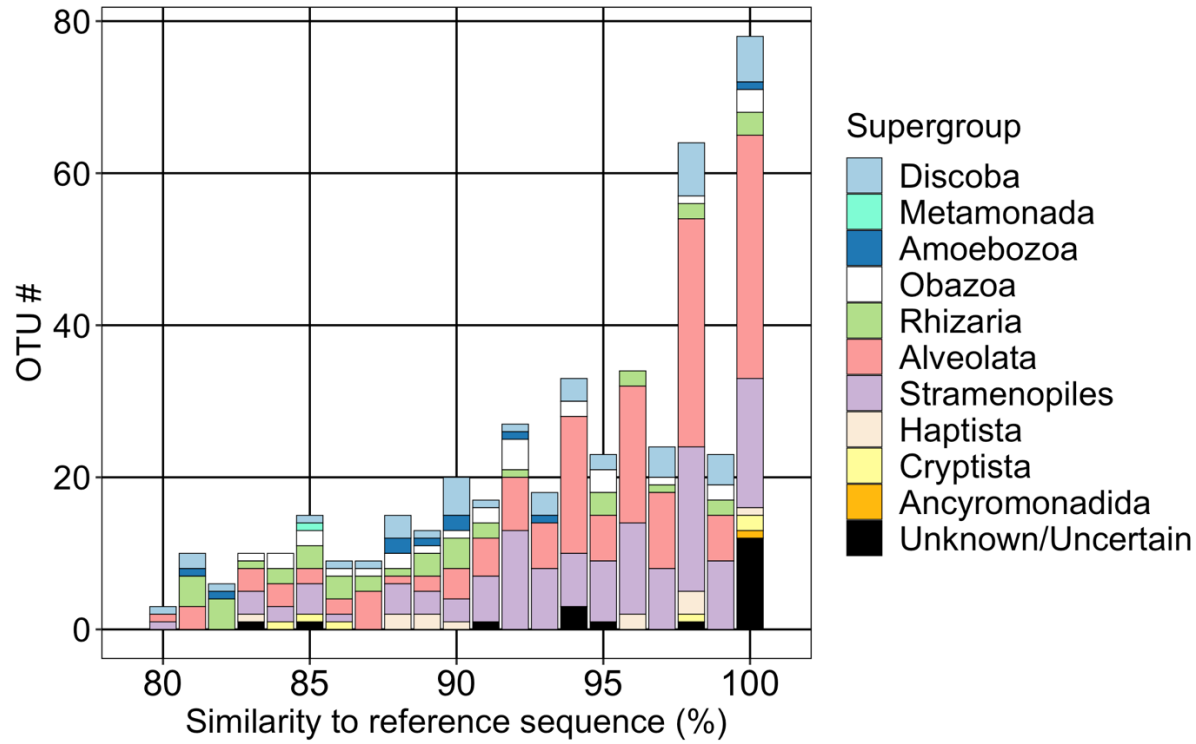

Figure S1. Biodiversity of OTUs of heterotrophic protists' within all salar samples. Similarity to reference sequences with an identity of >80 %.

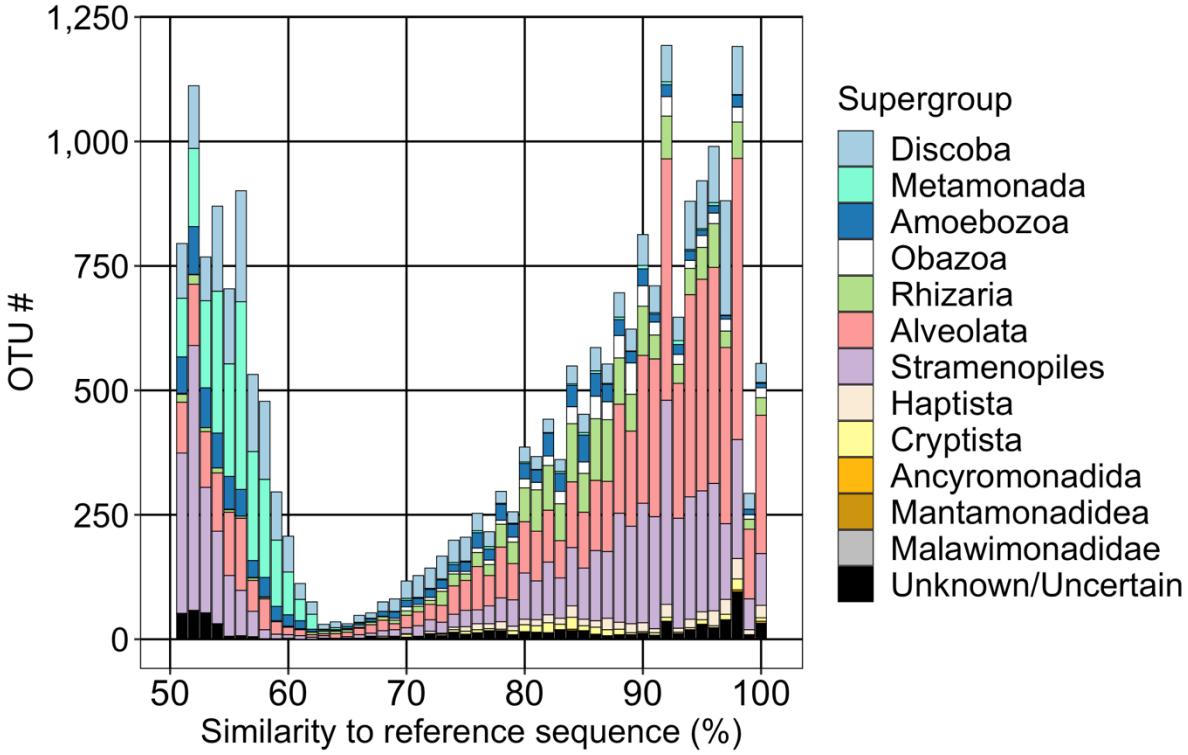

Figure S2. Biodiversity of OTUs of heterotrophic protists' within all salar samples. Similarity to reference sequences with an identity of >50 %.

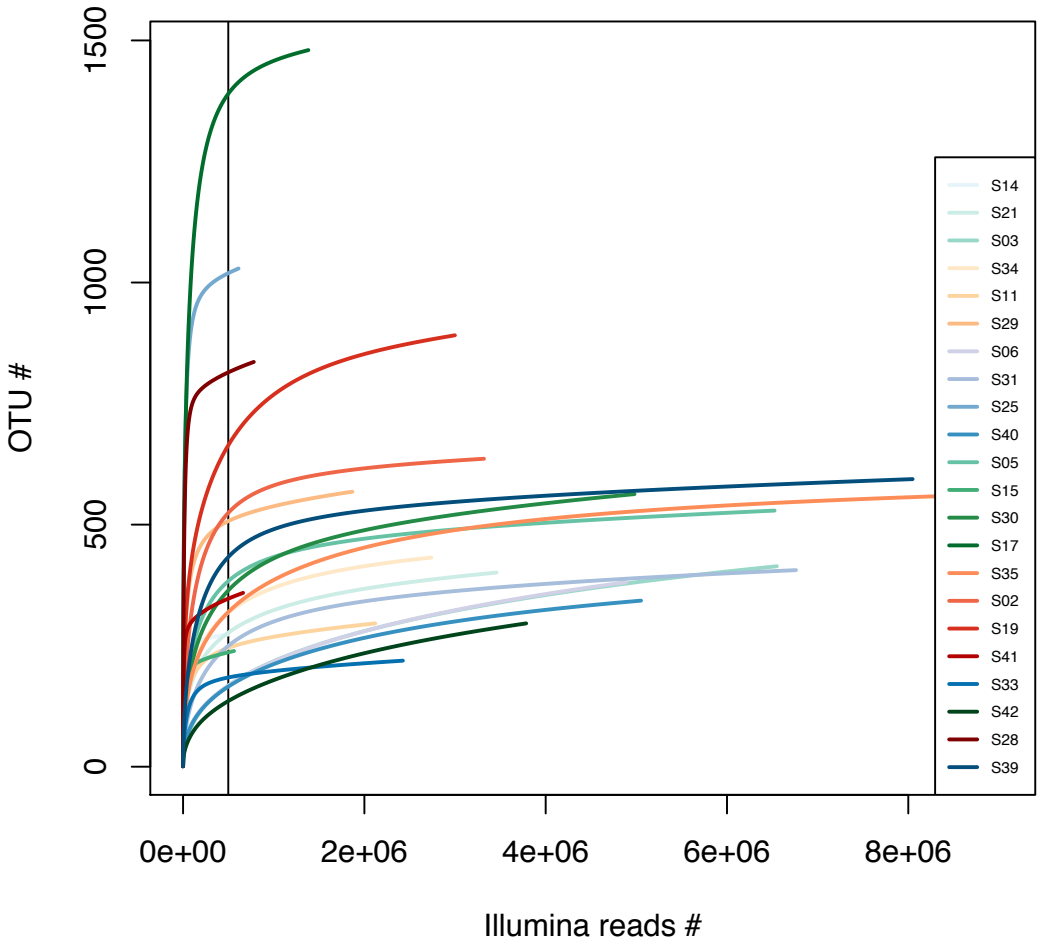

Figure S3. Rarefaction curve of all salar samples. Chimera were removed, as well as sequences with a quality value > 0.0001, reads < 87 bp, OTUs p-identity < 97 %.

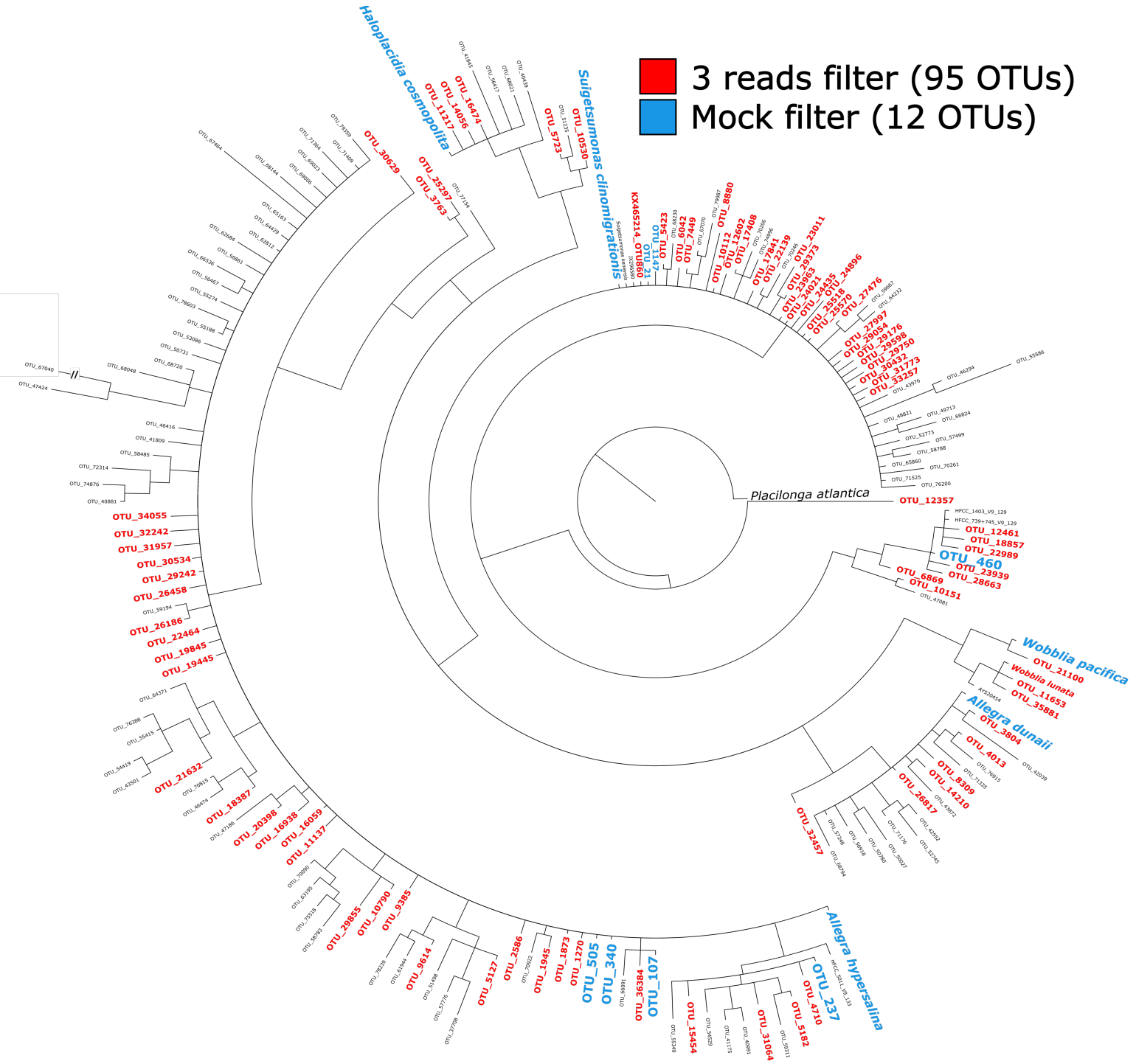

Figure S4. Biodiversity of Placididea. All OTUs of placidids are shown before the filtering (black,red and blue together). After filtering according to Schoenle et al. (2021), blue and red labelled OTUs remained. After mock community filter only blue OTUs were left.

Part 2

Biodiversity and distribution of aquatic protists in extreme environments of the Atacama Desert

Chapter 5

Biodiversity and distribution of protists via the water cycle in the Atacama - metabarcoding analyses of various natural water transport systems in northern Chile

Biodiversity and distribution of protists via the water cycle in the Atacama - metabarcoding analyses of various natural water transport systems in northern Chile

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Abstract

The transport of protists is possible due to formation of relatively small resting stages (cysts). In this way they can survive changing nonoptimal conditions or a temporary dry out. Especially rivers, fog and groundwater could possibly connect isolated salars and make an exchange between populations possible. To get a better overview of the associated protistan diversity and richness, the V9 region (SSU rDNA) of environmental samples from 15 different locations of water transport systems like groundwater, rivers, fog, marine water, as well as one geyser in the northern part of Chile was investigated. Coastal rockpools were additionally investigated as potential microhabitats separated from the marine water with a recurring exchange. The different habitats were mainly dominated by Alveolata and Stramenopiles and showed a mostly unique eukaryotic community at each location and only a small overlap between sampling sites. Rockpools showed to be relatively isolated microhabitats with potential water supply influenced by marine spray and coastal fog. Placididea were used as a model group to investigate these distribution patterns. For example, representatives of the genus *Allegra* were distributed mainly through groundwater, fog and the geyser. Marine and rockpool species (from the genus *Wobblia*) showed to be unique to their marine systems. These results suggest that only a small exchange of protists via these natural transport systems occurs and that the species are especially adapted to their original habitat.

Keywords: Metabarcoding, Placididea; V9 SSrDNA; protist distribution; eDNA; natural water transport systems, marine, rockpools, rivers, Stramenopiles; Heterotrophic flagellates, Atacama Desert

Introduction

In deserts, biological activity is generally restricted by the availability of water (Zeglin et al., 2011). One of the driest places worldwide, the Atacama Desert, offers a unique place for the investigation of aquatic environments (Berger and Cooke, 1997; Ericksen, 1983; McKay et al., 2003; Risacher et al., 2003; Clarke, 2006; Azua-Bustos et al., 2017), since most of the different water bodies are relatively isolated and separated by inhospitable conditions. Moisture generation within this region is rare and occurs along the coast through the formation of coastal fog due to the cold Humboldt Current below the inversion layer (Cereceda et al., 2002; Houston and Hartley, 2003; Cereceda et al., 2008). The "camanchaca", how high-altitude fog events are called, occurs nearly every day during winter times and only occasionally in the summer and is intercepted by the mountain ranges of the Chilean coast (occurrence between 400 - 1100 m) (Schemenauer et al., 1988; Cereceda et al., 2002). In some regions in the desert, this fog provides the only water source (Schemenauer et al., 1988) and is the most important source of humidity along the coastal range (Rundel et al., 1991; Pinto et al., 2006). At higher elevations (altiplano), moisture generation occurs due to precipitation of rain or snow. It creates a few perennial and ephemeral rivers crossing the Atacama Desert, mostly as groundwater below the surface (Houston, 2002, 2006). Rivers, in general, connect the landscape, including soils and groundwater, with the atmosphere and the oceans (Battin et al., 2008). Especially regarding ephemeral rivers, only a few studies investigated microbial communities and microbial diversity and distribution patterns are only poorly understood (Bunn et al., 2006; Zárate et al., 2020). The groundwater, coming from the east, also supplies the in the desert located permanent, mostly hypersaline inland water bodies, called salars (Demergasso et al., 2004). Metabarcoding studies about protist community composition of various salars already found that salars located in the Atacama Desert form separated systems

and contain unique eukaryotic communities with potentially highly adapted organisms (Stramenopiles and Alveolata) (Rybarski et al., in prep). This suggested that only a small exchange between these separated systems and allopatric speciation might have taken place. The relatively low dispersal rate and the ability of many protist species for a relatively fast genetic adaptation at high generation times suggest that the salars should be considered as hot spots of protist evolution. Due to the formation of resting stages (cysts), and their relatively small size, protists could possibly be transported into new habitats as well as survive changing nonoptimal conditions, e.g. the extremely high increase in salinity or habitats that temporarily dry out (Patterson, 1999; Rogerson and Detwiler, 1999; Figueroa et al., 2011). Regarding the distribution patterns of protists, there two partly contradicting hypotheses. On the one hand, it is assumed that they are globally ubiquitously dispersed via winds, birds, ocean currents and have only low allopatric speciation (Finlay and Clarke, 1999; Finlay and Fenchel, 1999; Finlay, 2002). On the other hand, there is the assumption that many microorganisms have their one biogeography and are particularly adapted to their specific habitat (moderate endemicity model) (Foissner, 2006, 2007). Within this study, we wanted to get more information on the possible transport mechanisms of protists and the potential exchange among protist communities of different habitats under the specific conditions of the Atacama Desert with its separated aquatic systems. Especially rivers, fog and groundwater could possibly connect different salars and make an exchange between populations possible. To get a better overview of the hidden protistan diversity and richness, the V9 region (SSU rDNA) of environmental samples from 15 different locations of covering groundwater, perennial and ephemeral rivers, fog, marine water, and rock pools, as well as one geyser in the northern part of Chile were investigated. A mock community was used as a reference community to keep sequencing errors and the overestimation of taxa richness to a minimum. In an earlier study, Placididea (mainly representatives from the

genus *Allegra*) have proven to be a suitable model group for analysing distribution patterns using NGS in hypersaline environments of the Atacama (Rybarski et al., in prep), and were therefore also examined as an important group in this study of distribution patterns in natural water transport systems.

Material and Methods

Sampling and DNA isolation of filter samples

Water samples were classified regarding their salinity according to Hammer (1986). Samples with a salinity of 0 PSU (practical salinity units) were considered freshwater, 0.5-3 PSU subsaline, 3-20 PSU hyposaline, 20-50 PSU mesosaline and > 50 PSU. Water was filtered from various water transport systems like groundwater, rivers, fog, marine waters, as well as one geyser from the northern part of Chile during several expeditions (Table 1, Figure 1). Filtration was performed with a suction filter holder (Sartorius Japan) and a polycarbonate filter (47 mm, 0.45 μm). Filters were transferred immediately into CryoTubes™ (Thermo Fisher Scientific, Waltham) and covered completely with 2 ml of ethanol (samples from 2019) or with a DMSO/EDTA/NaCl solution (DESS) for preservation (samples from 2018) (Gray et al., 2013; Yoder et al., 2006). Additional water sample for the identification of chemical parameters were taken in the year 2019. Parameters, of exact geographic location together with the associated coordinates, the altitude and the concentration of source salinity are shown in Table 1. Before DNA extraction, filter samples preserved in ethanol were placed in a heating chamber (70-80 °C) for ethanol removement. DESS samples were vortexed for at least 2 min, centrifuged (4000 *g*, 4 °C, 20 min) and the supernatant was discarded. DNA extraction was performed directly within CryoTubes™ (Thermo Fisher Scientific, Waltham) using the Quick gDNA ™ Mini Prep Kit (Zymo Research Corporation, CA, USA). 2100 μl of Genomic Lysis Buffer was applied on the filters, vortexed for 2 min and

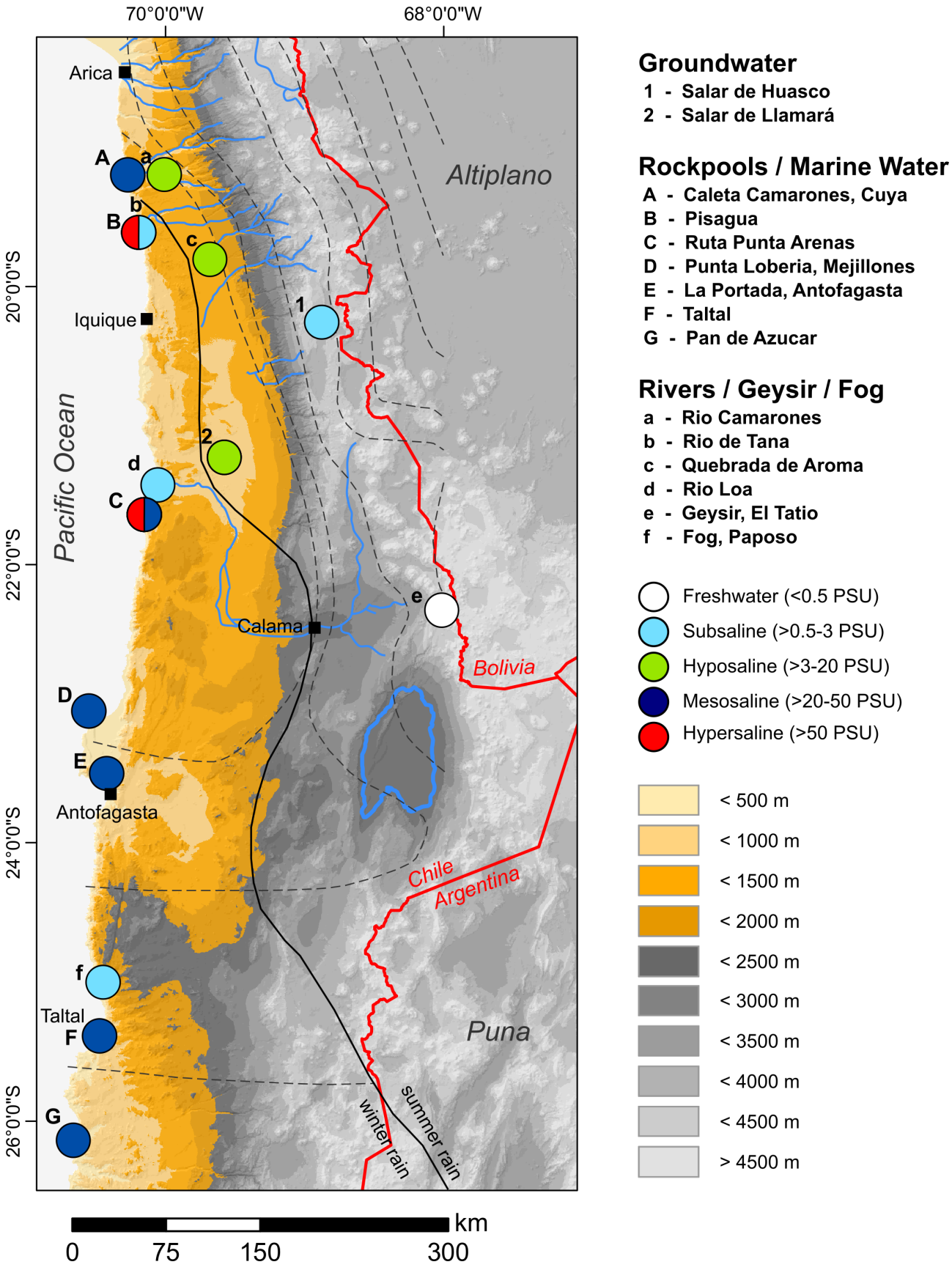

Figure 1. Map of sampling points in northern Chile. Colours of the positions indicate their salinity, several colours at one position demonstrate multiple samples with different salinities. More information on each sample can be found in Table 1.

centrifuged (4000 *g*, 4 °C, 15 min). Tubes were vortexed again and incubated at room temperature for 15 min. Further steps were performed applying the manufacturers' protocol except for additional washing step. The quality of each sample and the amount of DNA was measured via a Nanodrop Spectrophotometer ND-1000 (Peqlab, VWR, Erlangen, Germany) and stored afterwards at -23°C.

Principlal methods of DNA isolation and sequencing of the mock community, Amplification of V9 region, library preparation and sequencing, Demultiplexing and clustering into operational taxonomic units (OTUs), data analyses and chemical analyses were carried out as in Chapter 4.

Results

High-throughput sequencing of fresh- and brackish inflow samples

The mock community was evaluated separately in the same way like in Rybarski et al., (in prep., Chapter 4) The sequencing of 18 water samples resulted in 205,747,092 raw reads with both adapter-tagged primers, on average with 11.4 ± 6.9 million per sample. After removing reads that were too short, we ended up with 114,461,977 reads (111,936 OTUs), on average with 6.3 ± 3.9 million per sample. After removing chimera, sequences with a quality value > 0.0001, reads < 87 bp, OTUs p-identity < 80%, we ended up with 39,123 eukaryotic operational taxonomic units (OTUs) (99,722,568 reads) and at a 97% identity threshold were 5,284 (82,731,368 reads). Each of all samples showed a nearly saturated rarefaction curve with slopes in the range of 0 (S22) to 5.35×10^{-4} (S10) (Table S1). The alpha diversity, calculated via the Shannon index ranged from 0.06 to 4.09, with a mean value of 2.05 (Table S1). Removing Metazoa, Fungi, Streptophyta and phototrophic organisms (OTUs p-identity < 80%), resulted in 29,429 OTUs (58,516,927 reads) which could be assigned to heterotrophic protists (Table S3). The average amplicon size of the V9 region of

Table 1. Information of filtered water samples, including the exact geographical location with the associated coordinates, the altitude, the salinity, and the volume of filtered water. Which Mids were used, and the measured ionic composition for each sample are also listed here.

	Sampling Location		Filter	Lat/Long	Salinity [PSU] / Altitude [m]	Sample	Volume [ml]	Mid	Na [mmol/l]	K [mmol/l]	Ca [mmol/l]	Mg [mmol/l]	Cl [mmol/l]	S [mmol/l]	As [mmol/l]	B [mmol/l]	Ba [mmol/l]	Li [mmol/l]	P [mmol/l]	Si [mmol/l]	Sr [mmol/l]
1	Salar de Huasco	S14	Filter-18_029	20°15'45.1"S 68°52'33.4"W	1 / 3790	groundwater inflow	500	UDI_007	3.1	0.14	1.12	0.34	1.23	0.87	0	0.28	0	0	0	0.69	0
2	Salar de Llamará	S24	Filter-18_048	21°16'12.4"S, 69°37'19.8"W	4 / 744	groundwater inflow	250	UDI_008	29.6	0.84	18.0	1.7	38.65	18.19	0	0	0	0	0	1.34	0.03
		S13	Filter-19_011	21°13'24"S, 69°32'42"W	6 / 787	rain-run-off before salar	300	UDI_005	39.3	0.85	22.14	5.63	20.94	34.46	0	0.55	0	0	0	0.48	0.08
A	Caleta Camarones - Cuya	S44	Filter-18_034	19°11'49.35"S, 70°16'1.6"W	42 / 3	rockpool	200	UDI_012	-	-	-	-	-	-	-	-	-	-	-	-	-
B	Pisagua	S12	Filter-18_036	19°35'50"S, 70°12'52"W	90 / 1	rockpool	250	UDI_004	-	-	-	-	-	-	-	-	-	-	-	-	-
C	Ruta Punta Arenas	S08	Filter-19_007	21°38'28"S, 70°08'43"W	37 / 0	marine water	500	UDI_012	4025.91	93.42	0	609.96	4844.85	2203.02	0	0	0	0	0	0	0
		S32	Filter-19_009	21°38'27"S, 70°08'43"W	315 / 7	rockpool	50	UDI_007	487.63	10.27	13.23	55.86	668.49	34.97	0	0.59	0	0	0	0	0.10
D	Punta Loberia, Mejillones	S10	Filter-18_002	23°3'14.907"S, 70°32'57.984"W	40 / 0	marine water	500	UDI_002	-	-	-	-	-	-	-	-	-	-	-	-	-
E	La Portada, Antofagasta	S01	Filter-18_001	23°30'24.3"S, 70°25'26.0"W	40 / 0	marine water	500	UDI_002	-	-	-	-	-	-	-	-	-	-	-	-	-
F	Taltal	S04	Filter-18_008	25°23'24.76"S, 70°28'25.72"W	40 / 0	marine water	500	UDI_005	-	-	-	-	-	-	-	-	-	-	-	-	-
G	Pan de Azucar	S16	Filter-19_002	26°08'27"S, 70°39'45"W	36 / 0	water	500	UDI_011	463.76	9.35	13.45	49.67	536.99	38.50	0	0.56	0	0	0	0	0.08
a	Rio Camarones	S20	Filter-18_039	19°12'01"S, 70°00'38"W	5 / 366	water	250	UDI_003	43.34	0.35	16.53	1.49	67.83	20.53	0	1.46	0	0	0	0.7	0.02
b	Rio de Tana	S38	Filter-18_041	19°33'17.79"S, 70°12'8.49"W	2 / 17	water	150	UDI_003	-	-	-	-	-	-	-	-	-	-	-	-	-
c	Quebrada de Aroma	S07	Filter-18_032	19°48'23"S, 69°40'41"W	4 / 1266	water	500	UDI_011	48.49	2.17	3.85	1.59	49.59	5.07	0.02	5.54	0	1.05	0	0.72	0.03
d	Rio Loa	S37	Filter-19_008	21°25'41"S, 70°03'15"W	2.5 / 0	water	300	UDI_002	-	-	-	-	-	-	-	-	-	-	-	-	-
e	El Tatio	S26	Filter-18_015	22°19'53.2"S, 68°00'45.1"W	0 / 4271	water from geyser	250	UDI_012	3.22	0.39	0	0.01	2.42	0.14	0.02	0.33	0	0	0.15	1.99	0
f	Paposo	S23	Filter-19_014	25°00'29'' S, 70°27'01"W	1 / 711	fog collection 1/2	100	UDI_007	-	-	-	-	-	-	-	-	-	-	-	-	-
		S22	Filter-19_015	25°00'30'' S, 70°27'01"W	1 / 710	fog collection 2/2	50	UDI_005	3.21	0.65	0.54	0.39	34.59	0.57	0	0	0	0	0.16	0.02	0

18S rDNA sequences was 126 bp (± 7 bp, standard deviation). 3-reads filter resulted in 17,028 OTUs (58,502,530 reads). The samples S01, S04, S07, S08 were additionally filtered by the mock S09; S10, S12, S13, S14, S16 by mock S18; S20, S22, S23, S24, S26 by mock S27; S32 by mock S36 and S37, S38, S44 by mock S45, respectively. Appling the additional filter of the mock community to the dataset ended up with 779 OTUs (56,051,470 reads) of heterotrophic protists. On average, unfiltered OTUs could be assigned to reference sequences with a p-identity of 74 % (Figure S2). 25.7 % of all filtered OTUs could be assigned with 100% identity to a reference sequence from the database of which 5.9 % of OTUs were unknown or uncertain. 42 % of OTUs could be assigned to sequences with a similarity of 98 %. Most filtered OTUs from 80-100% similarity to reference sequences could be assigned to Alveolata (49.2 %) and Stramenopiles (20.7 %) (Figure S1). Within the Stramenopiles, 1.2 % of OTUs could be assigned to Placididea.

Community composition

The calculation of the beta diversity of all 18 samples from various aquatic environments in the Atacama Desert, formed four separated clusters. The first cluster was represented by only one groundwater inflow sample from the Salar de Llamará (S24). Groundwater inflow into the Salar de Huasco (S14), Quebrada Aroma (S07) and Rio Camarones (S20) formed the second cluster. The third cluster comprises primarily of samples from marine water (S08 - Ruta Punta Arenas, S04 – Taltal, S10 - Punta Loberia, Mejillones) but also a sample from a river (Rio de Tana - S38) and a rockpool (Caleta Camarones - S44). The last cluster contains various samples from nine different sampling locations. The first three clusters were moderately supported (bootstrap = 0.74-0.88), the fourth and largest cluster was not well supported (bootstrap = 0.5) (Figure 3).

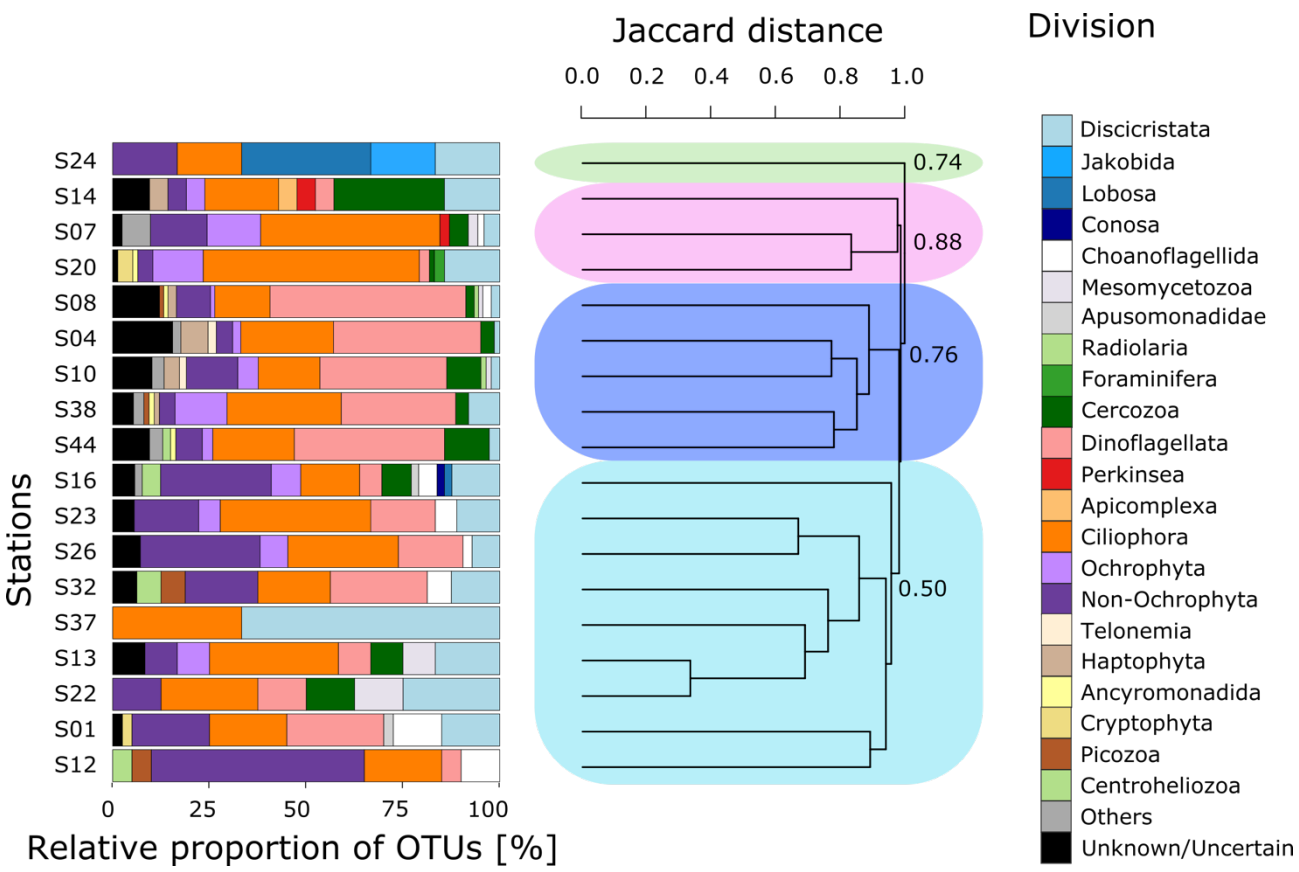

Figure 2. Community composition and clustering of inland water samples. Relative proportion of OTUs was assigned to taxonomic groups. Others - Relative proportion of OTUs <1 %; Unknwn/Uncertain - OTUs could not be assigned to a taxonomic group. Clusters of the dendrogram, based on the Jaccard index, are highlighted and bootstrap values are given for each cluster.

The samples itself were mainly dominated by Stramenopiles (39.1 % Ochrophyta and 60.9 % non-Ochrophyta), Ciliophora (51.4 % of Alveolata). Ochrophyta were mainly represented by the class of Chrysophyceae (90.5 %), Stramenopiles (non-Ochrophyta) by the MAST group (29.6 %), Bicosoecida (25.5 %), Labyrinthulea (23.5 %), Placididea (9.2 %) and Oomycota (7.1 %). Ciliophora are mainly represented by Oligohymenophorea (29.9 %), Spirotrichea (43.1 %), Litostomatea (7.6 %) and Phyllopharyngea (8.1 %). In some samples Cercozoa (7.3 % of all filtered OTUs) and Dinoflagellata (22.3 % of all filtered OTUs) have also a high abundance (S14) as well as Discicristata (5.8 % of all filtered OTUs, of which 28.9 % can be assigned to Heterolobosea

and 71.1 % to Euglenozoa). The third clade contains many samples with unknown or uncertain OTUs.

Similarity of heterotrophic protists' OTUs between locations regarding the salinity concentration

Both different filter approaches were conducted and illustrated against each other. Subsaline to hyposaline samples from river systems (Rio Camarones (a), Rio de Tana (b), Quebrada de Aroma (c) and Rio Loa (d)), subsaline to hyposaline (groundwater inflows, fog collection, rain run-off from Andes), mesosaline (marine water) as well as hypersaline (rockpools) samples were compared to each other (Figures 1, 3-6). There were no OTUs shared between all different river samples regarding the mock community filter (Figure 3A). Rio de Tana (41 %) and Quebrada Aroma (30 %) contained the highest amount of unique OTUs. Rio Loa shared only a low amount of OTUs between all other samples and owned no unique OTUs. Rio Camarones shared OUTs with every other river, most OTUs with the sample from Quebrada Aroma (5 %). Nearly the same pattern of distribution could also be found in the deep sea sediment (Schoenle et al., 2021b) (Figure 3A'). There were no OTUs shared between all different subsaline to hyposaline samples regarding the mock community filter (Figure 4A). Rain run-off from the Andes before the Salar de Llamará shared most OTUs with the fog collection samples from Paposo. Groundwater inflow from Salar de Huasco showed most unique OTUs (43.8 %). Nearly the same pattern of distribution could also be found in the deep sea sediment (Schoenle et al., 2021b) (Figure 4A') but there were 2 % of all OTUs shared between the samples. Fog collection samples (1/2) and groundwater inflow from the Salar de Huasco contained most of the unique sequences within this filtering approach. All different mesosaline samples shared also no OTUs regarding the mock community filter (Figure 5A). The sample from Taltal and Punta Loberia, Mejillones shared most OTUs within both filtering approaches. Punta Loberia

Mejillones contained most unique OTUs within both filtering methods. Samples from hypersaline rockpools shared 3% of all OTUs within both filtering approaches (Figure 6). Every sample also showed its unique community with only low overlap. Both filtering methods showed nearly the same pattern but OTUs of the rockpool with the highest salinity and the mesosaline one shared with the mock community filter only 1 %, within the method after Schoenle et al., 2021b 5 % (Figure 6A,A').

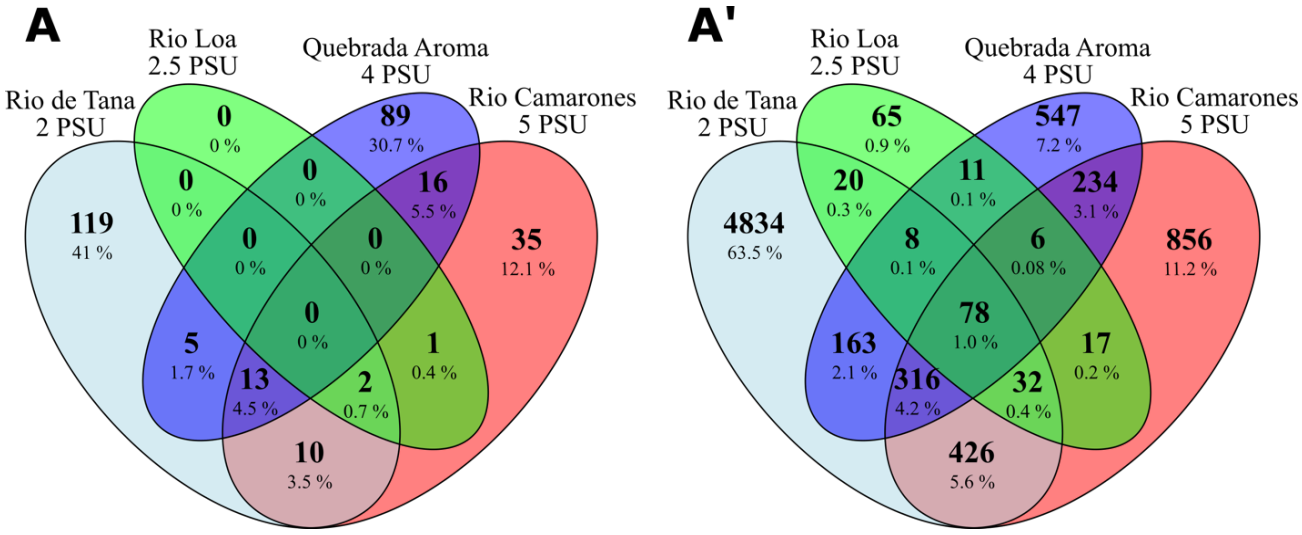

Figure 3. Comparison of heterotrophic protists' OTUs of samples from subsaline to hyposaline conditions. Venn diagrams show the number and percentage of unique and shared OTUs. A were filtered regarding the mock community; A' were filtered regarding Schoenle et al. (2021).

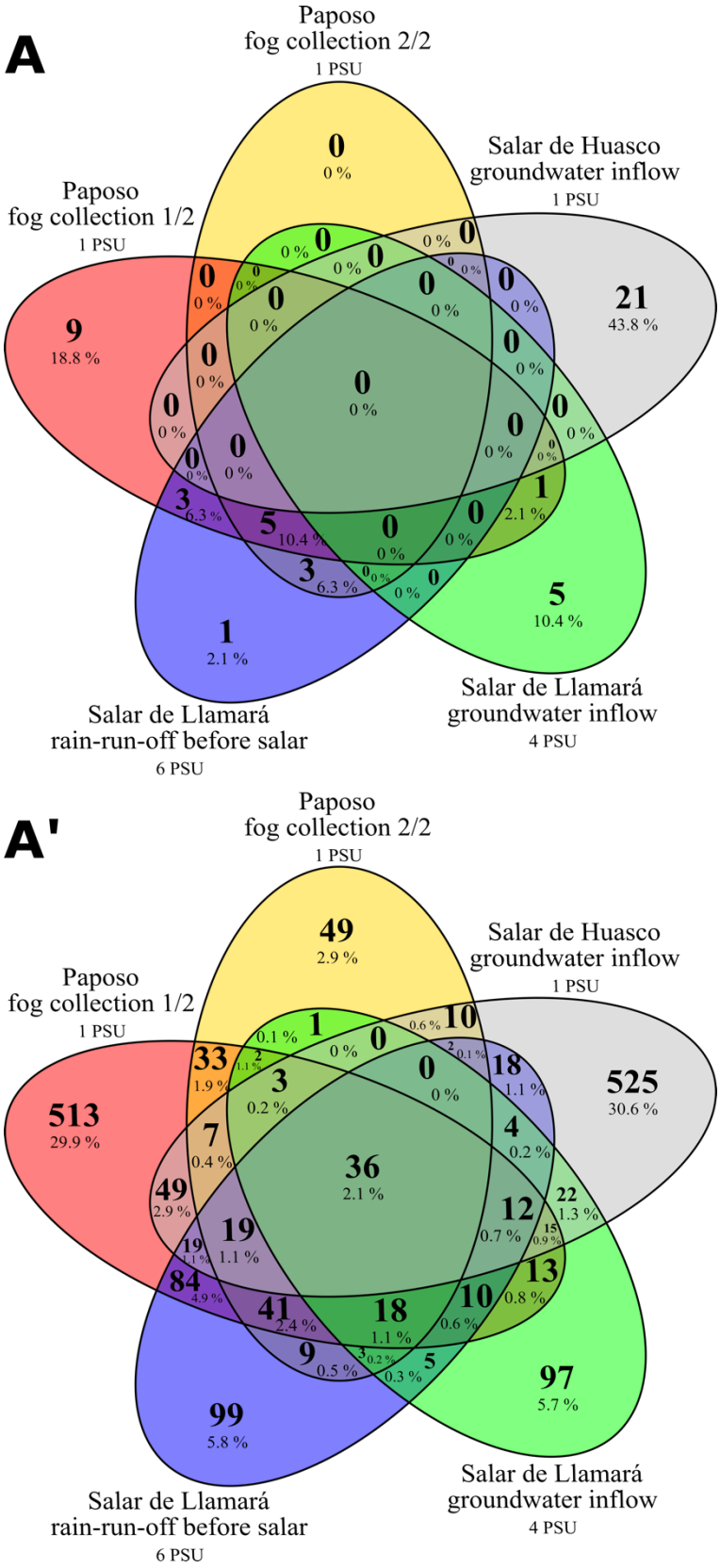

Figure 4. Comparison of heterotrophic protists' OTUs of samples from subsaline to hyposaline conditions. Venn diagrams show the number and percentage of unique and shared OTUs. A were filtered regarding the mock community; A' were filtered regarding Schoenle et al. (2021).

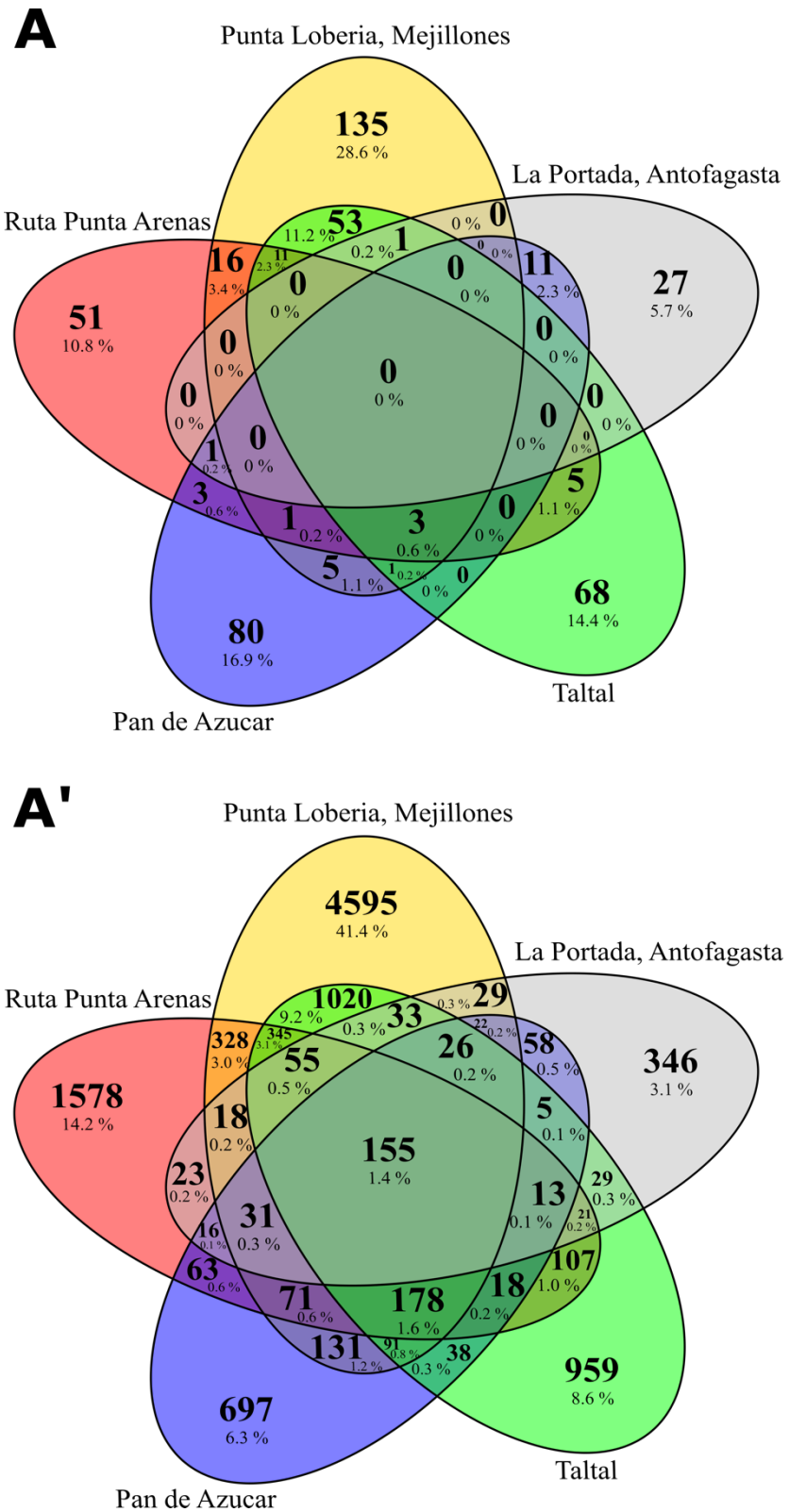

Figure 5. Comparison of heterotrophic protists' OTUs of samples from mesosaline conditions. Venn diagrams show the number and percentage of unique and shared OTUs. A were filtered regarding the mock community; A' were filtered regarding Schoenle et al. (2021).

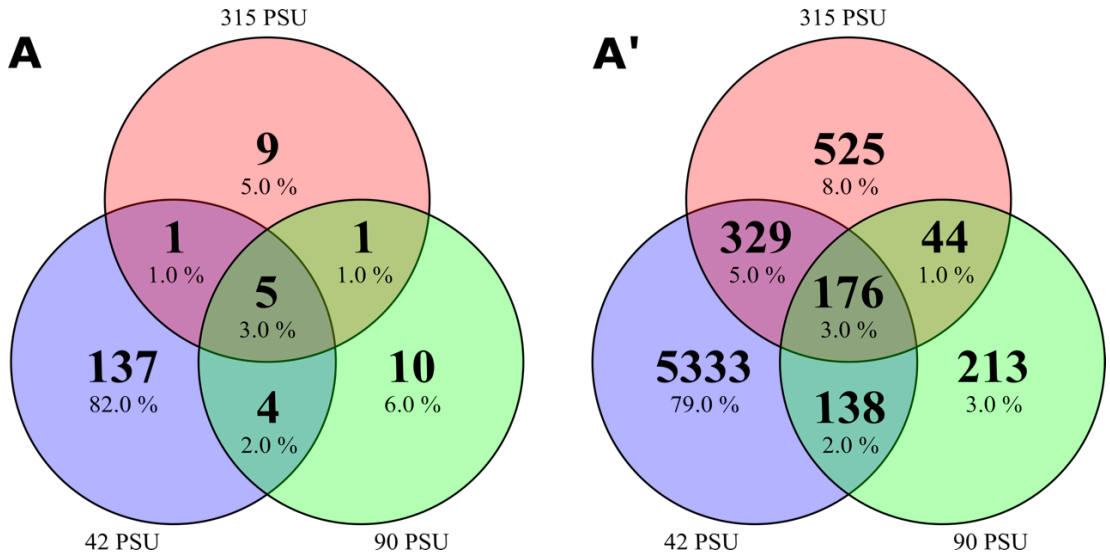

Figure 6. Comparison of heterotrophic protists' OTUs of samples from hypersaline (rockpool) conditions. Venn diagrams show the number and percentage of unique and shared OTUs. A were filtered regarding the mock community; A' were filtered regarding Schoenle et al. (2021).

The comparison of biodiversity in terms of geographical location

In this approach also both different filter steps also were conducted and illustrated. The comparison of samples regarding their adjacent locations showed that no OTUs were shared between all of them and most OTUs were unique to the samples (Figures 7-9). Each of the samples to the north (Figure 7) also contained its own community composition and in both filtering methods, only a small amount of OTUs overlapped. This pattern could also be observed at the middle position (Figure 8) and the groundwater, geyser and Quebrada Aroma compassion (Figure 9). The salinity was related to the OTU composition of all samples within a NMDS plot (Figure 10). The NMDS plot showed that all marine samples, freshwater to subsaline from (fog collection, Rio Loa and rain run-off from the Andes) samples as well as hyposaline river samples clustered together regarding their OTU composition. Two rockpool sample (S12, S44) clustered together with the marine samples the other (S32) clustered with the subsaline samples, where also the fog samples clustered. Samples from the groundwater inflow cluster outside all other samples (Figure 10, S14, S24).

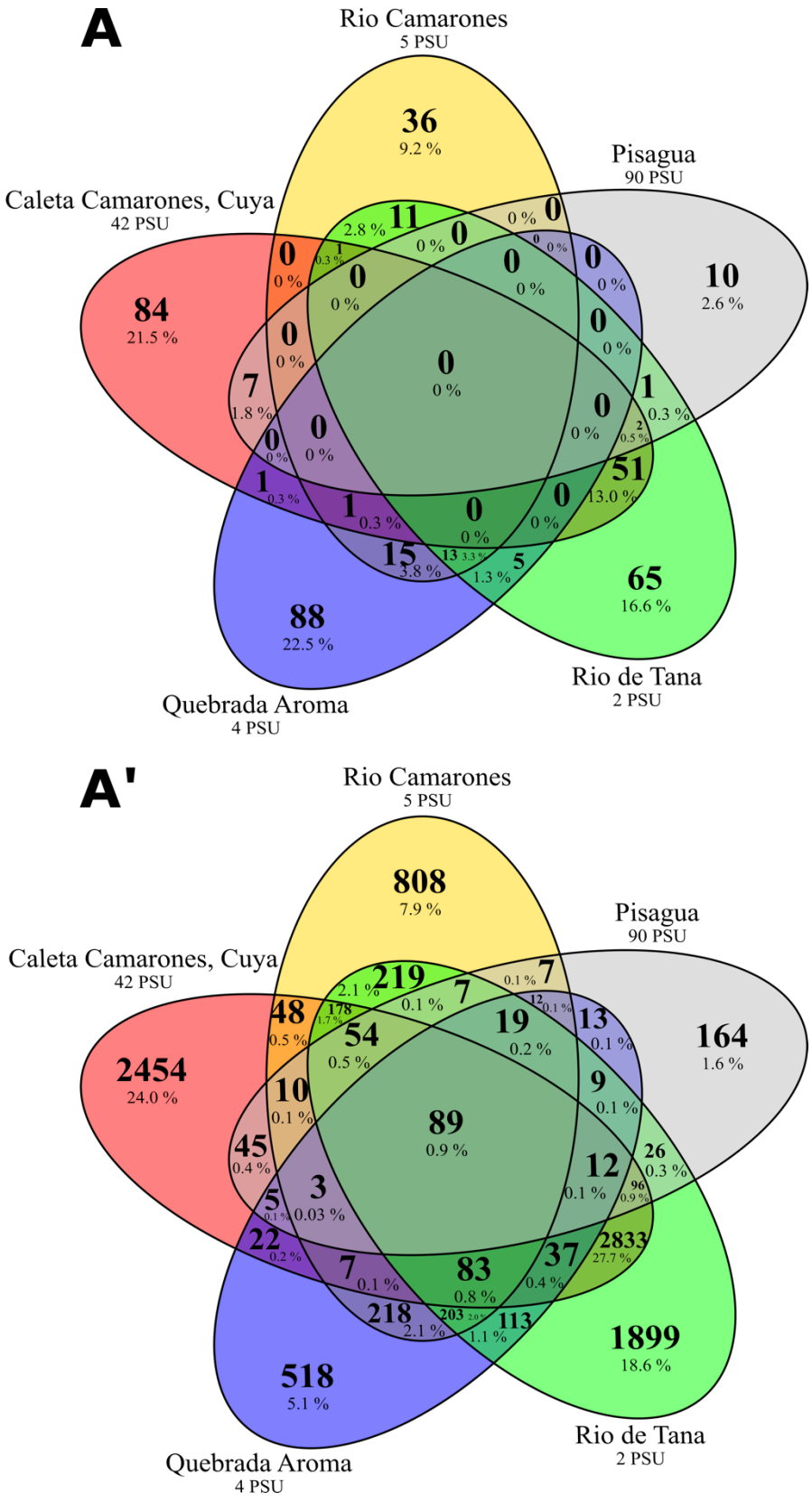

Figure 7. Comparison of heterotrophic protists' OTUs of samples from adjacent locations (North). Venn diagrams show the number and percentage of unique and shared OTUs. A were filtered regarding the mock community; A' were filtered regarding Schoenle et al. (2021).

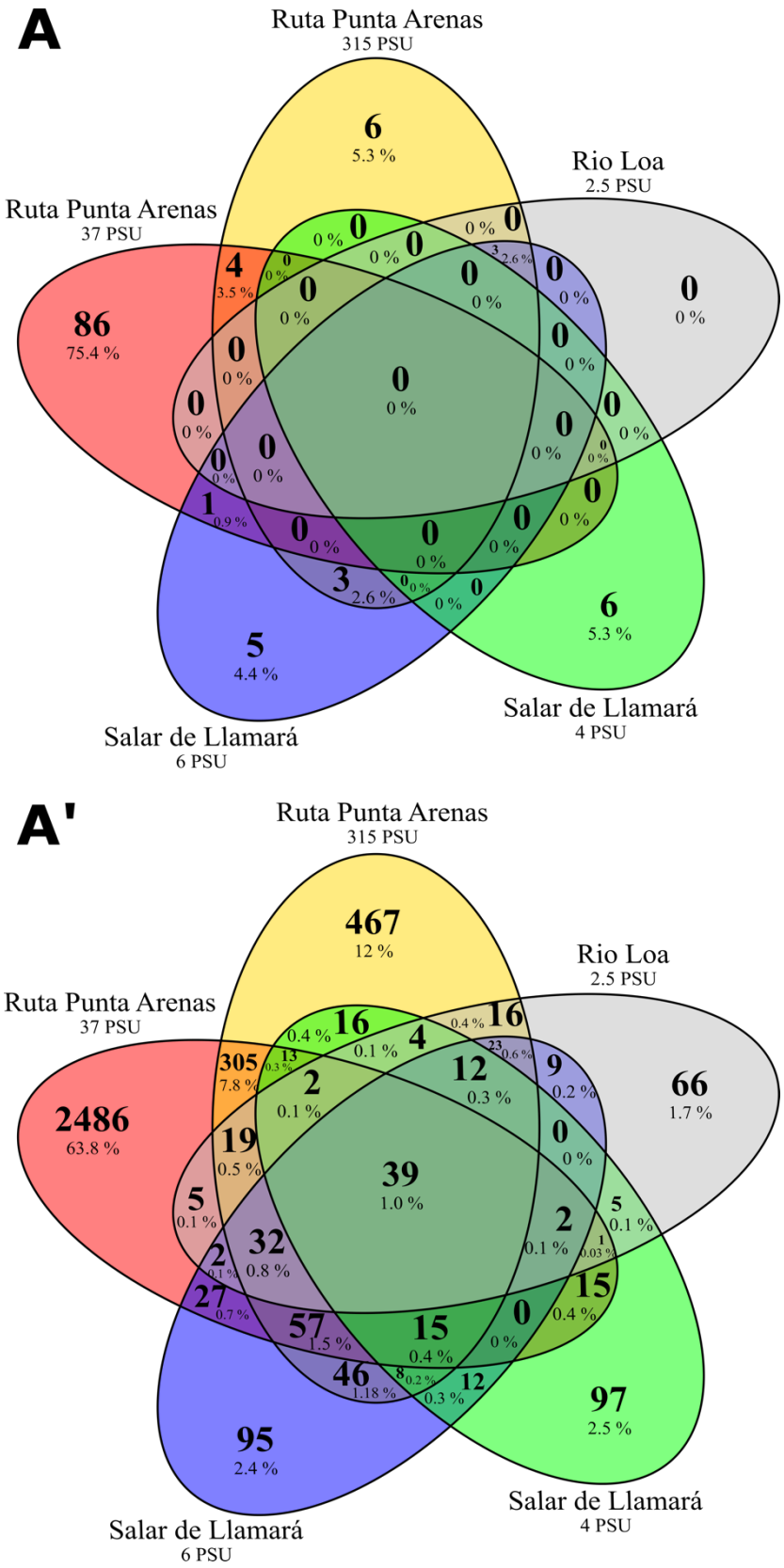

Figure 8. Comparison of heterotrophic protists' OTUs of samples from adjacent locations (middle) Venn diagrams show the number and percentage of unique and shared OTUs. A were filtered regarding the mock community; A' were filtered regarding Schoenle et al. (2021).

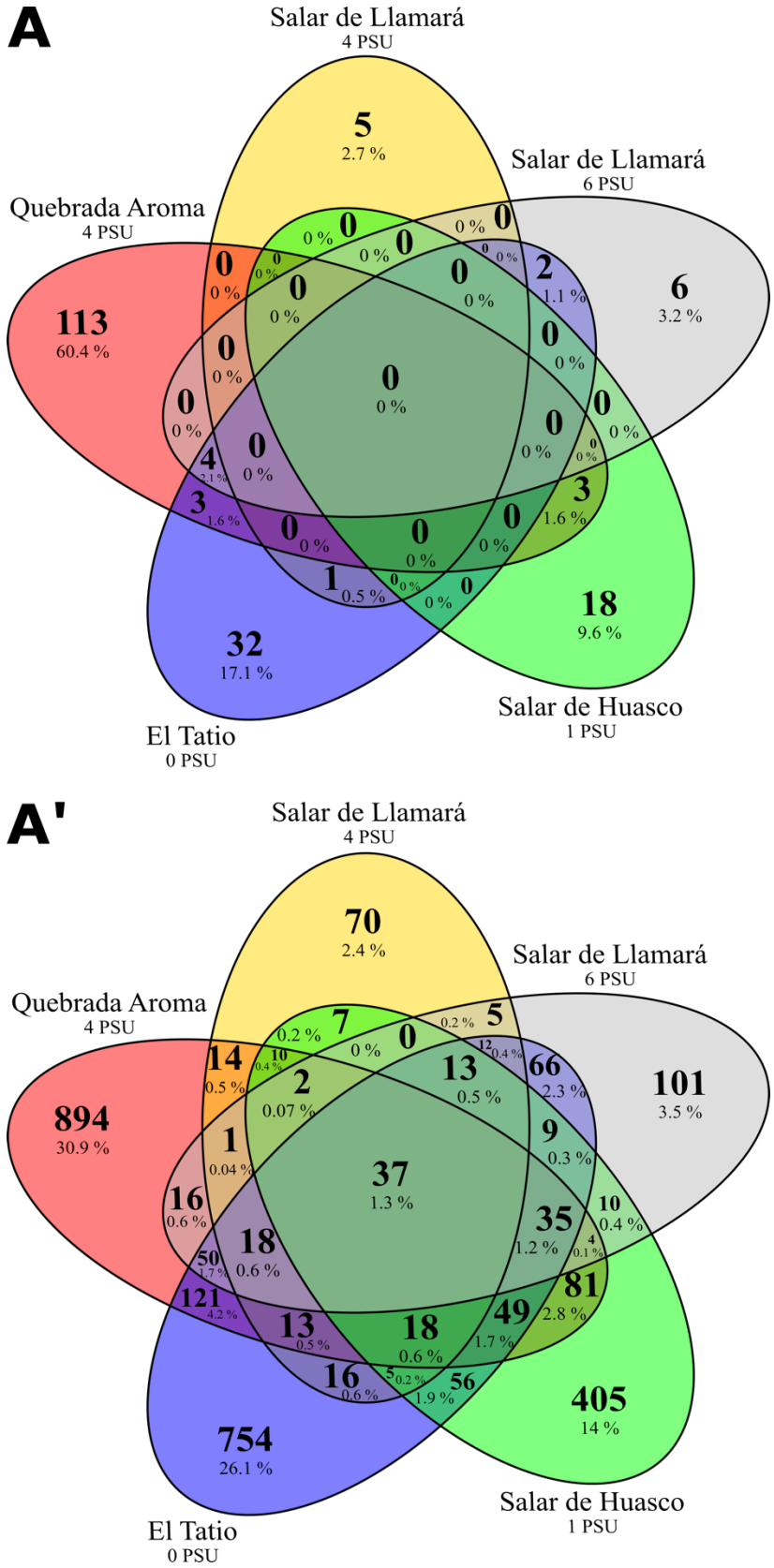

Figure 9. Comparison of heterotrophic protists' OTUs of samples from adjacent locations. Salar de Llamará groundwater to the Salar de Llamará (4 PSU) and to the Salar de Huasco (1 PSU); rain run-off from the Andes before the Salar de Llamará (6 PSU); Quebrada Aroma and the geyser at El Tatio. Venn diagrams show the number and percentage of unique and shared OTUs. A were filtered regarding the mock community; A' were filtered regarding Schoenle et al. (2021).

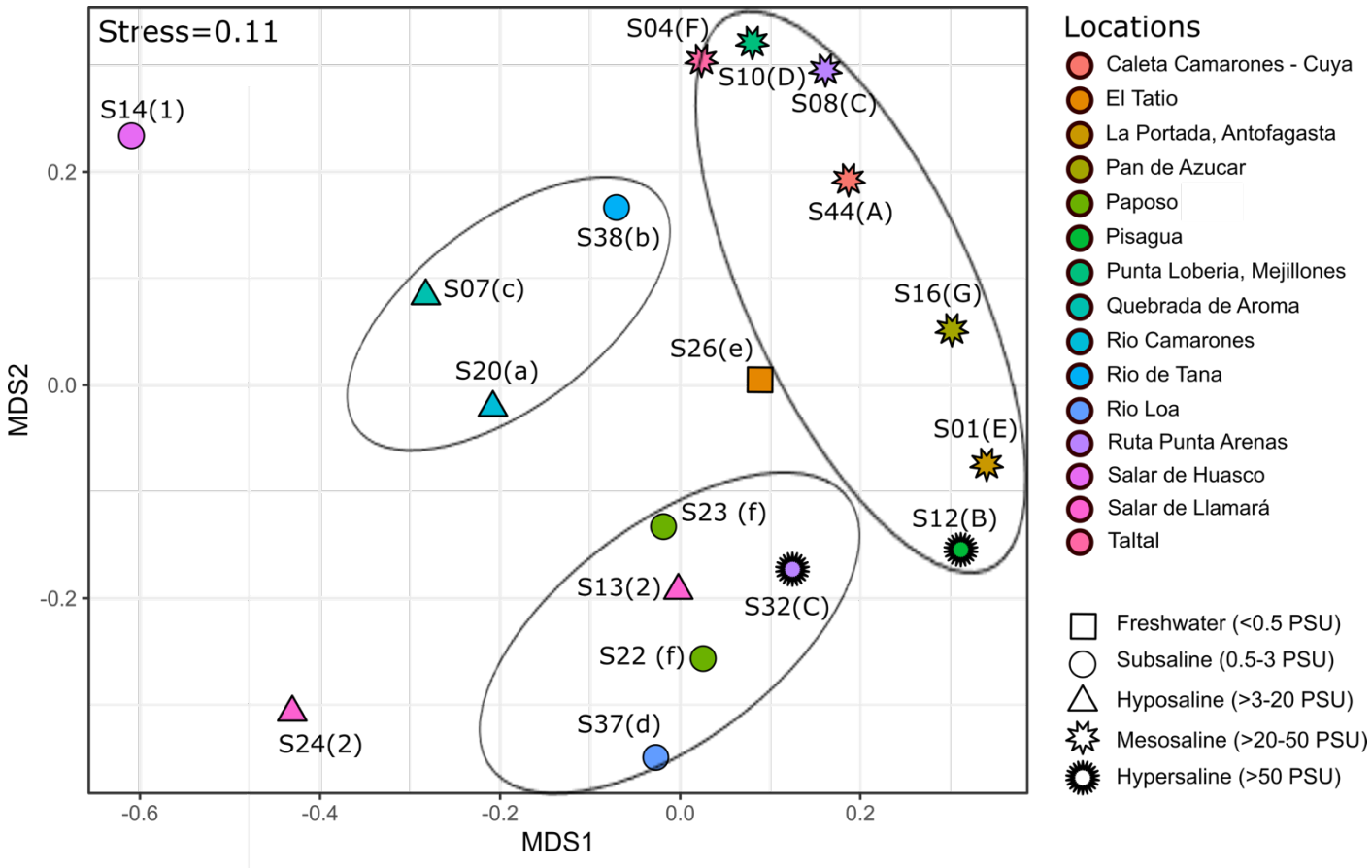

Figure 10. Non-metric multidimensional scaling (NMDS) plot based on the Jaccard Index. Different symbols indicate the classification regarding salinity concentration. Circles indicate a possible grouping regarding the community composition and the salinity.

Chemical composition

To relate the metabarcoding data to the habitat, we also investigated the concentration of different ions (Figure 11). In all different samples, the dominant ions were sodium and chlorine. Groundwater inflow samples as well as the rain run-off before the Salar de Llamará showed a relatively high proportion of calcium and mainly the marine samples (S08; S32; S16) of magnesium. Silicon (Si) occurred in one sample from the geyser (S26) in relatively high concentrations. Sulfur occurred also in the groundwater inflows as well as in one marine water sample in higher concentrations. The sample from El Tatio also showed the highest concentration of potassium

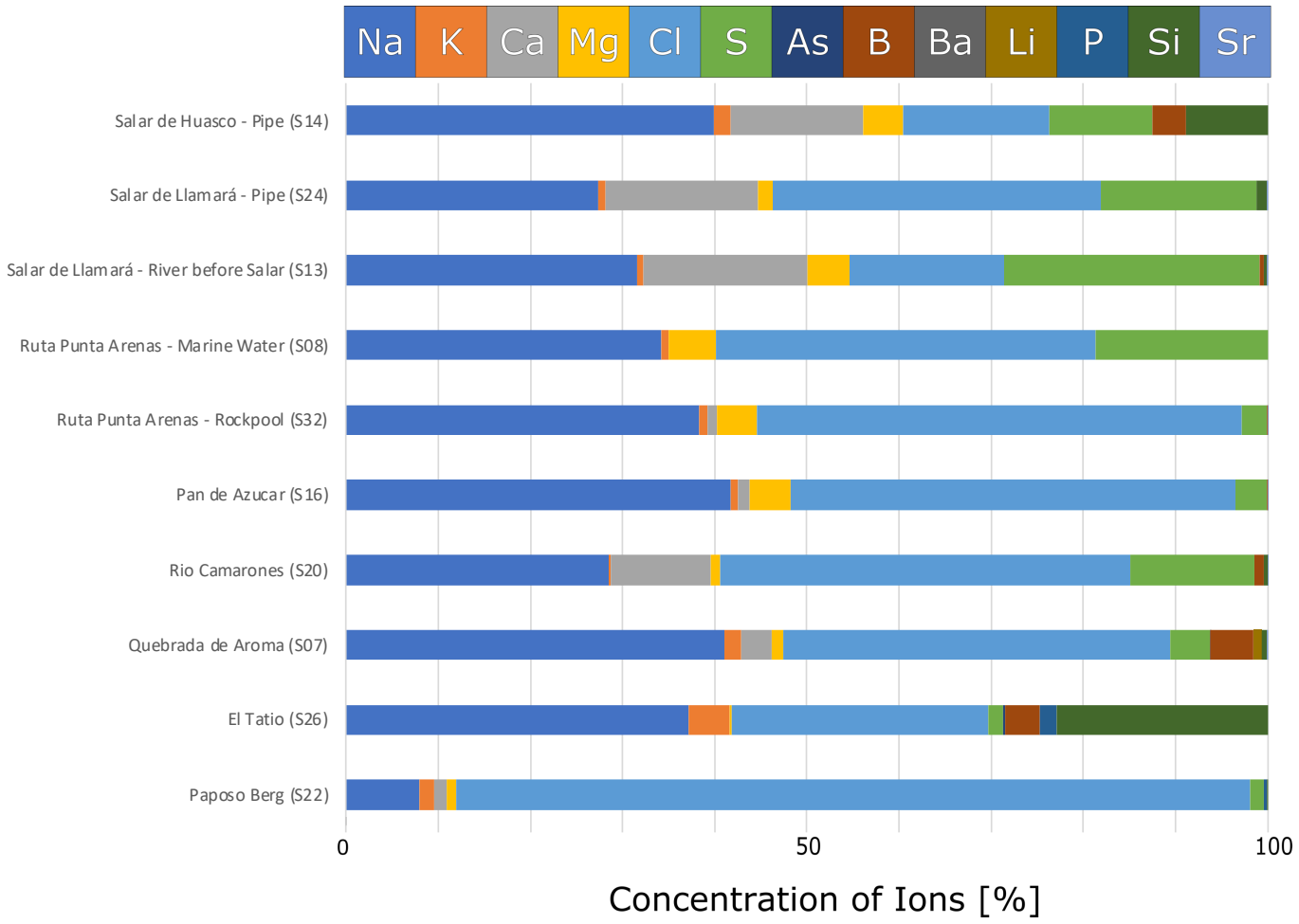

Figure 11. Relative proportion of the ionic composition investigated via the Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) for most salar samples. Exact concentrations of ions can be found in Table 1.

We checked the presence of OTUs belonging to the class of Placididea in all samples (Figure 12). Only OTUs with an identity of 100 % to a known placicid species were included. Placidids were detected in seven different samples. The genotype of *Allegra hypersalina* occured detected in three different samples: the groundwater inflow to the Salar de Llamaá (S24), a fog collection sample (S23) and a geyser sample (S26). The genotype of *Allegra dunaii* was only found in one geser sample (S26). The genotype of *Wobblia pacifica* was localized within marine and hypersaline waters (S01 - La Portada, ; S12 – Pisagua; Antofagasta; S44 - Caleta Camarones – Cuya). The genotype of Wobblia lunata was only found in one marine water sample at Pan de Azucar (S16).

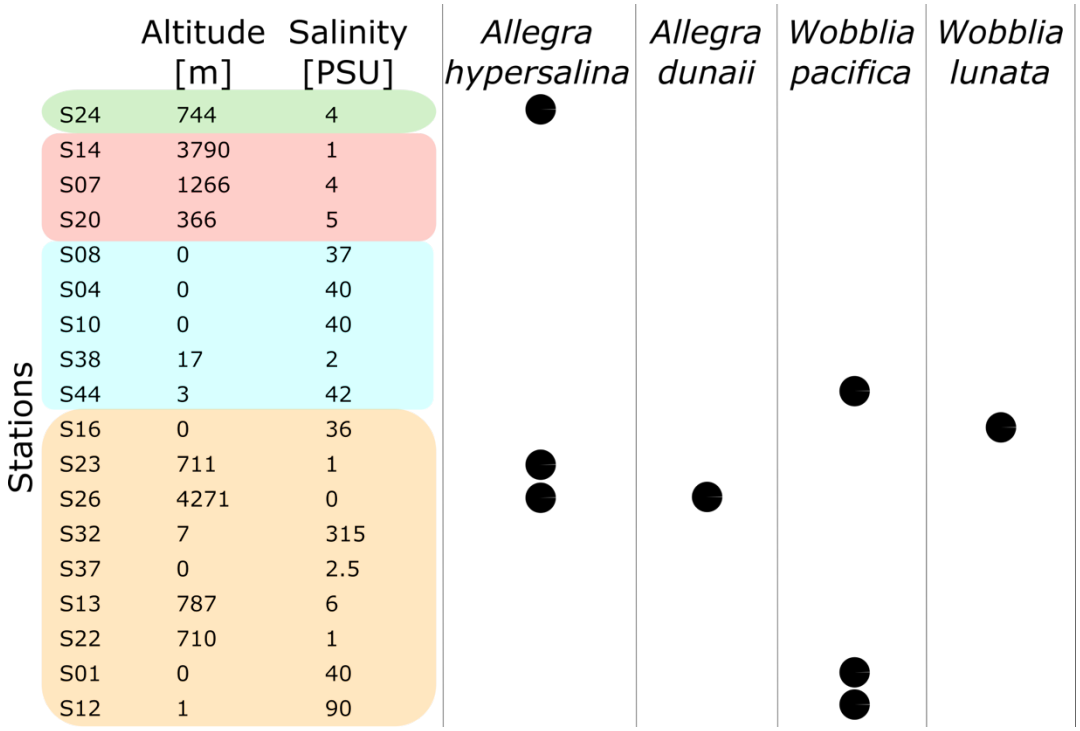

Figure 12. Genotype identification of Placididea within the different samples. The mock community filtered data was used for this identification. Black dots indicate the presence of this genotype in the sample with 100 % identity. Coloured clusters indicate the clusters that were also obtained by the Jaccard clustering.

Discussion

This study investigated the protists' communities of 15 different locations in the northern part of Chile. Various natural water transport systems like groundwater, rivers, fog collections, marine water, and one geyser in the altiplano, were investigated. Coastal rockpools were additionally sampled as potential microhabitats separated from the marine water with a recurring exchange. Due to their hypersaline conditions, they could potentially serve as a starting point for the colonization of hypersaline salars. The V9 region of 18S rDNA turned out to be a good tool to understand the diversity of the microbial community if the read number is taken into account (Choi and Park, 2020). Within our metabarcoding approach, we conducted two different filter

methods and compared them to each other. By applying a relatively strong read filter, where a mock community was used as a reference community, we tried to significantly reduce sequencing errors and the overestimation of taxa richness. However, the disadvantage of this method is that due to the strong filtering, a possible overlap could not be found, although it would potentially be there. Therefore, a second filtering was conducted already used in previous metabarcoding studies with a lower read filter (e.g. Schoenle et al., 2021). Comparing various natural water transport systems as well as rockpools, mostly unique eukaryotic communities were found at each location by applying the strong filter regarding the mock community and only a small overlap between sampling sites occurred. This suggested that only a small exchange of protists from other habitats via these transporting systems occurred. Previous metabarcoding studies showed that mainly Stramenopiles (Ochrophyta and non-Ochrophyta) and Alveolata (Ciliophora) were isolated from hypersaline environments in the Atacama Desert (Rybarski et al., in prep, Chapter 4). In our investigations of different aquatic non-salar habitats regarding natural water transport systems, representatives of Alveolata and Stramenpoiles showed also to be the dominant organisms. They seem to be especially adapted, also in these systems with lower salinity. But it shouldn't be forgotten that primers are not equally good for all eukaryotic groups (e.g. Cercozoen) which would also result in low representation of groups. Nevertheless, typical marine organisms have not been found in freshwater, which supports this method. Within this study, we wanted to get more information on the possible transport mechanisms of protists and the potential exchange among protist communities of different habitats under the specific conditions of the Atacama Desert with its separated aquatic systems. Metabarcoding studies about protist community composition of various salars already found that salars located in the Atacama Desert form separated systems and contain unique eukaryotic communities with potentially highly adapted organisms (Stramenopiles and Alveolata) (Rybarski et al., in

prep, Chapter 4). Especially rivers, fog and groundwater could possibly connect different salars and make an exchange between populations possible. The comparison of the different rivers showed that the geographically closer rivers, such as Rio Camarones, Rio de Tana, and Quebrada Aroma share more genotypes than the Rio Loa, for example (Figure 3). On the other hand, Rio de Tana and Quebrada Aroma also contained a relatively high unique community composition. Quebrada Aroma and Rio Camarones share most of the genotypes, which could be due to their close location in the Central Depression (Pampa del Tamarugal), and thus, an exchange due to potentially connected groundwater systems, wind or fog could be possible (Risacher et al., 2003; Hoke et al., 2004). The community composition of closely located sites (Figure 7) showed that the marine rockpool sample Caleta Camarones, Cuya (A) and the sample of the river Rio Tana (a), which is in the direct vicinity, also have a relatively high proportion of shared genotypes. However, it is again the case that all different locations contain a high proportion of unique OTUs, indicating only little exchange between them. The groundwater inflow at Salar de Huasco located in the altiplano showed the highest amount of unique OTUs and no overlap to the other subsaline to hyposaline samples (Figure 4). This could be explained due to its isolated location in the altiplano. Compared to the other, mainly coastal sampling locations like the fog and the rain run-off close to the Salar de Llamará, it is difficult to imagine that there is an exchange due to the Coastal Cordillera. Even in the previous studies conducted by Rybarski et al. (in prep, see also Chapter 4), the Salar de Huasco did not share any genotypes with the Salar de Llamará, and only a low amount of OTUs was shared between several different salars located within the altiplano. Therefore, the Salar de Huasco could be seen as a separated system with a unique community composition and only low possible exchange. On the other hand, samples from habitats close to the Salar de Llamará and the fog seem to share a small proportion of genotypes. Some studies detected fog in this region (Cereceda et

al., 2002). It could be possible, that the rain run-off from the Andes close to the Salar de Llamará possibly entered, due to very rare flooding events. Thus, a small but infrequent exchange would be possible. The marine samples show a similar pattern (Figure 5). Unique community compositions were found at each location where marine water was sampled, and only little overlap occurred between the different sampling positions. The rockpool S32 located at position C clustered with the samples from the fog, the river before the Salar de Llamará. It could be possible that the fog influenced this sample, and no exchange with the marine habitat occured. Also, the associated marine water shared only a small amount of OTUs with this rockpool (Figure 8). It is possible that even at the coast isolated microhabitats with especially adapted organisms occur with a unique community composition. These organisms tend to be adapted to varying and rising salinities due to evaporation. This study also used placidids as a model group to analyse and get more information about potential global distribution patterns. Mainly genotypes of the representatives of the genus *Allegra* could be identified in this metabarcoding approach. We found this genotype only in the groundwater of the Salar de Llamará, the fog and the geyser. This also supports the assumption of a potential exchange, and *Allegra* could be possibly transported via fog or groundwater. The Rio Loa, near the Salar de Llamará, shares only 3% of the OTUs with the rain run-off from the Andes before the Salar de Llamará. No placidids have been found in the Rio Loa, which suggests that this river does not play an essential role in spreading placidids. The placidid genotype of *Wobblia pacifica* was detected within rockpools and from a marine water sample, suggesting that species from the rockpools are closely related to other marine species. The genotype of the marine species *Wobblia lunata* could only be detected in marine coastal waters. This observation was also phylogenetically supported by representatives of the Atlantic Ocean, Japan and the Caribbean Sea, which formed a marine clade at the base of the phylogenetic tree, reflecting the potential marine origin of

placidids. In summary, these investigations showed a possible distribution of placidids via natural transport systems and a unique community composition at all different systems with only a low overlap. There is a possibility of low exchange and dispersal with possibly highly adapted organisms within unique communities. Enormous diversity of the individual habitats makes the Atacama particularly interesting and unique for understanding the evolution and distribution of protists. Rare flooding events connect the Andes salars possibly via groundwater or like the rain run-offs before the Salar de Llamará.

Author contributions

Sampling in the Atacama Region was carried out by A.E.R., F.N., and H.A.; DNA isolation was carried out by A.E.R.; A.E.R. and A.S. performed bioinformatical analyses of the data. A.E.R., C.V. and M.S. performed investigations of chemical parameters. The project was launched and supervised by H.A.; A.E.R. and H.A. wrote the manuscript. All authors reviewed and revised the manuscript.

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Supplementary Material

Biodiversity and distribution of protists via the water cycle in the Atacama - metabarcoding analyses of various natural water transport systems in northern Chile

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Table S1. Slopes of rarefaction curve and Shannon index calculated for all samples, as well as the mean, the maximal and the minimal value.

	slope	Shannon index
S01	9,05E-05	0.8
S04	3,52E-04	1.65
S07	1,03E-04	4.01
S08	2,96E-04	1.92
S10	5,35E-04	4.09
S12	1,10E-04	1.06
S13	2,67E-05	1.2
S14	6,41E-05	2.43
S16	1,41E-04	3.26
S20	1,81E-04	2.23
S22	0,00E+00	1.48
S23	1,07E-04	1.2
S24	5,23E-05	0.06
S26	8,15E-05	2.96
S32	1,93E-04	1.05
S37	2,03E-05	0.61
S38	4,70E-04	3.08
S44	4,22E-04	3.87
min	0	1
max	5,35E-04	4.09
mean	1,80E-04	2.05

Table S2. Filter steps of the OTUs (A) and reads (B). Chimera were removed, as well as sequences with a quality value > 0.0001, reads < 87 bp, OTUs p-identity < 80% from the OTU table, we ended up with 29,429 heterotrophic protists' operational taxonomic units (OTUs). A - Number of OTUs together with OTU loss; B - Number of reads together with reads loss. Heterotrophic protists were then filtered due to the mock community resulting in 779 OTUs (56,051,470 reads) and according Schoenle et al. (2021) (3 reads filter) resulting in 17,028 OTUs (58,502,530 reads).

A

Filter	Kept	NumOTUS	Proportion of total OTUs [%]	OTUs lost	OTUs lost [%]
None	**Total**	111936	100	0	0
NoHit	**Only Eukaryota**	100157	89.48	-11779	10.52
Chimeras	**Without Chimeras**	74166	66.26	-25991	25.95
Length	**larger 87 bp**	73212	65.41	-954	1.29
Quality	**smaller 0.0001**	53749	48.02	-19463	26.58
p_idenity	**80-100%**	39123	34.95	-14626	27.21
Exclude Fungi		37813	33.78	-1310	3.35
Exclude Metazoa		34919	31.2	-2894	7.65
Exclude Streptophyta		34697	31	-222	0.64
Phototrophic Protists	**Heterotrophic Protists**	29429	26.29	-5268	15.18
Mock reads filter	**Heterotrophic Protists**	779	2.65	-28650	97.35
3 reads filter	**Heterotrophic Protists**	17028	57.86	-12401	42.14

B

Filter	Kept	NumReads	Proportion of total reads [%]	reads lost	reads lost [%]
None	**Total**	114461541	100	0	0
NoHit	**Only Eukaryota**	108825638	95.08	-5635903	4.92
Chimeras	**Without Chimeras**	108617967	94.89	-207671	0.19
Length	**larger 87 bp**	108611425	94.89	-6542	0.01
Quality	**smaller 0.0001**	106691518	93.21	-1919907	1.77
p_idenity	**80-100%**	99722568	87.12	-6968950	6.53
Exclude Fungi		99097364	86.58	-625204	0.63
Exclude Metazoa		69597248	60.8	-29500116	29.77
Exclude Streptophyta		69353364	60.59	-243884	0.35
Phototrophic Protists	**Heterotrophic Protists**	58516927	51.12	-10836437	15.62
Mock reads filter	**Heterotrophic Protists**	56051470	95.79	-2465457	22.75
3 reads filter	**Heterotrophic Protists**	58502530	99.98	-14397	0.13

Figure legends

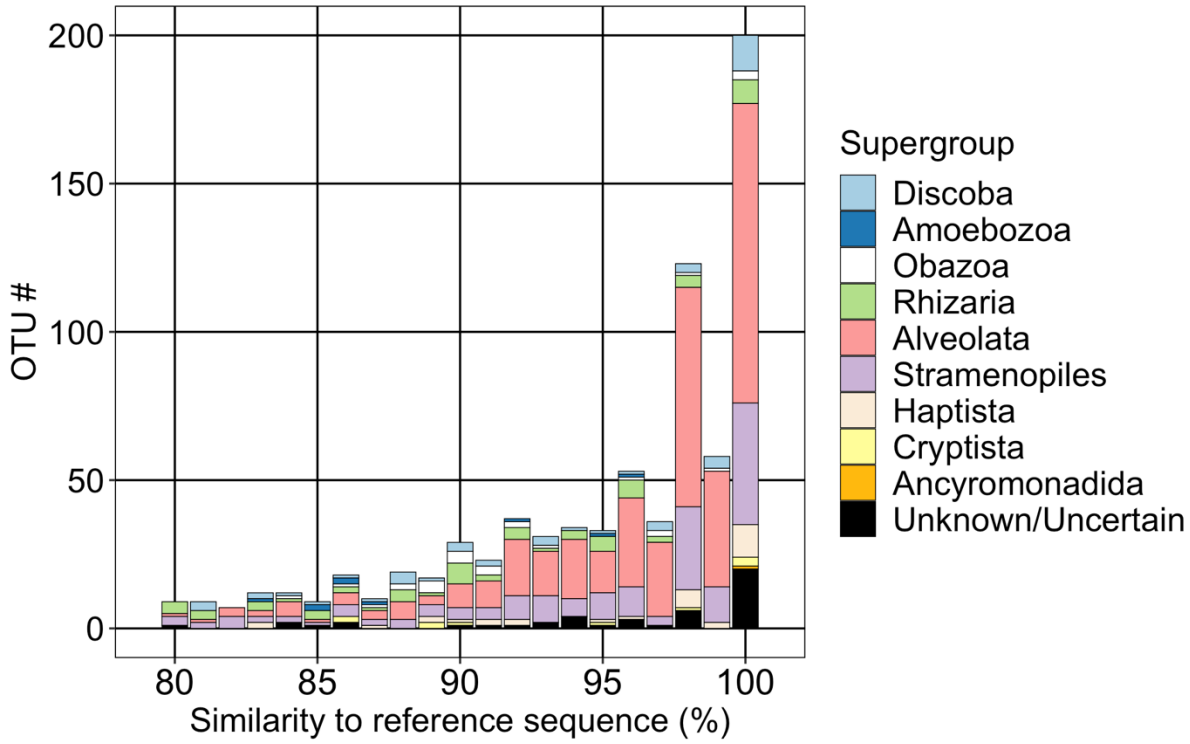

Figure S1. Biodiversity of OTUs of heterotrophic flagellates within all salar samples. Similarity to reference sequences with an identity of >80 %.

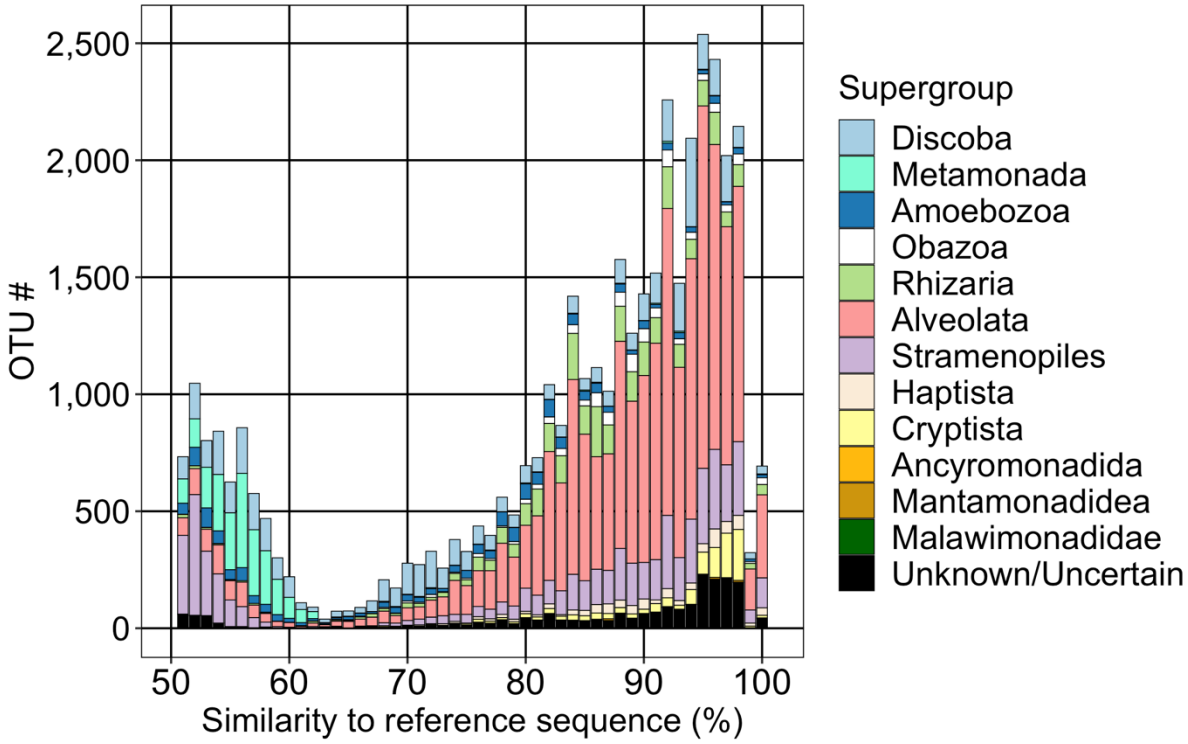

Figure S2. Biodiversity of OTUs of heterotrophic flagellates within all salar samples. Similarity to reference sequences with an identity of >50 %.

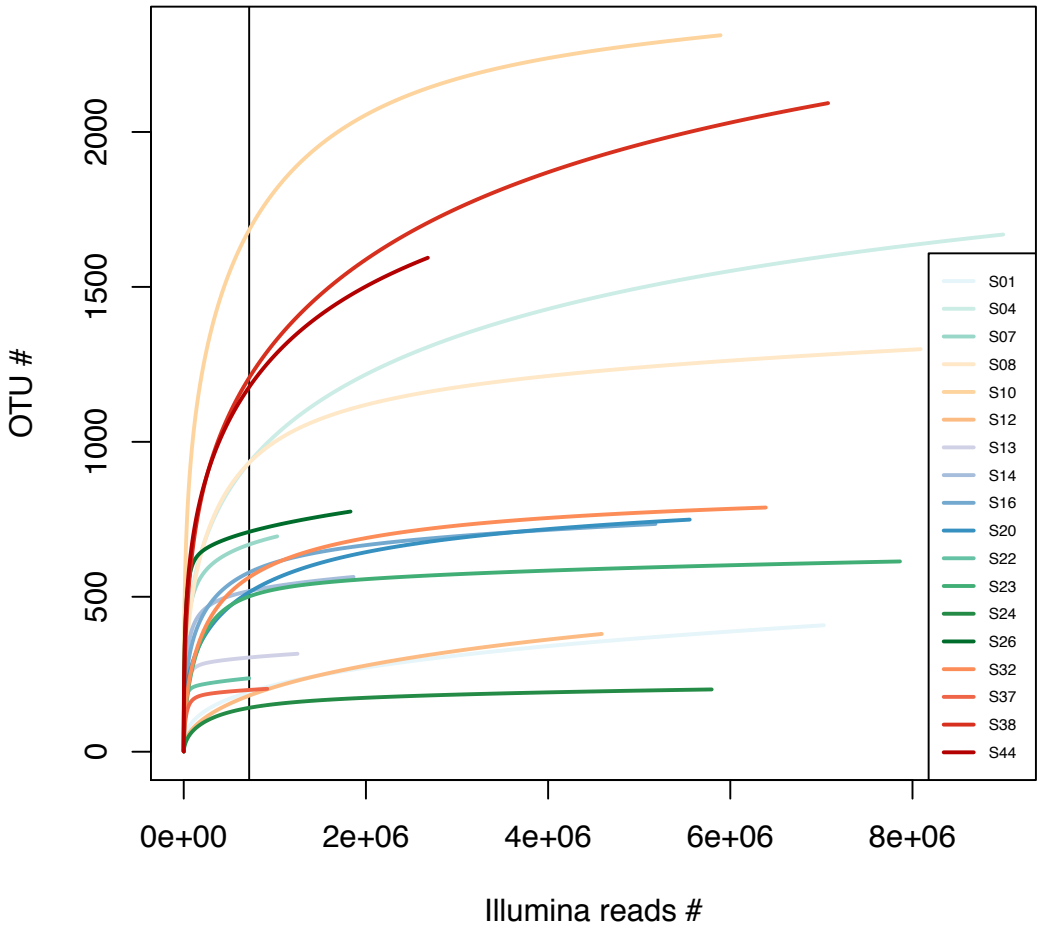

Figure S3. Rarefaction curve of all samples. Chimera were removed, as well as sequences with a quality value > 0.0001, reads < 87 bp, OTUs p-identity < 97 %.

Part 3

The Evolution of Protists

Chapter 6

Mirroring the effect of geological evolution: Protist divergence in the Atacama Desert

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Research article

Mirroring the effect of geological evolution: Protist divergence in the Atacama Desert

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ABSTRACT

Unicellular eukaryotes, also called protists, are potentially fast evolving organisms. The small size of protists, their fast reproduction rate and ability to form cysts as well as their adaptability to extreme conditions allow them to associate to endemic animals, plants, saline lakes and soil even in extremely arid systems. These properties make unicellular eukaryotes ideal model organisms to combine studies on evolutionary processes of very different groups of organisms and across very different time scales comprising even geological ones. The hyperarid Atacama Desert offers a study area unique on Earth, where a predominantly arid climate was present for millions of years.

A comprehensive analysis of the diversity of unicellular eukaryotes in different habitats (endemic desert plant phyllosphere, the gut of endemic darkling beetles, isolated hypersaline waters) revealed a dataset distinct and divergent from other regions on Earth. We used standard isolation and cultivation protocols to elucidate divergence patterns for a variety of very different and independent taxonomic groups of protists such as gregarines and ciliates among alveolates, placidids among stramenopiles and choanoflagellates among opisthokonts. The ability to rapidly adapt to extreme environments, which enhance a fast divergence rate at high UV radiation, has only been reported for prokaryotes up to now. The establishment of arid to hyperarid conditions in the Atacama Desert about 20 Ma ago has obviously led to an isolation of protist populations followed by a radiation of species. There are only a few regions on Earth with similar extreme salinity conditions reducing the chance of an exchange between protist populations. Divergence patterns in unicellular eukaryotes in very different phylogenetic groups independently mirror the effect of geological evolution and climate variability during the Neogene.

1. Introduction

1.1. Unresolved protist evolution

Recent fundamental studies of species diversity in surface waters of the oceans revealed protists (unicellular eukaryotes) to be the genetically most diverse group of organisms (e.g. de Vargas et al., 2015), which seems to be true also for terrestrial systems (Geisen et al., 2015; Venter et al., 2017). Still today, our understanding of the processes leading to this enormous diversity is rather poor. The high diversity of microbial eukaryotes forms an essential basis for understanding the evolution of multicellularity (King et al., 2008; Fairclough et al., 2013; Ratcliff et al., 2013). This is why protists have become important model organisms for experimental evolutionary studies (e.g. Fairclough et al., 2013; Ratcliff et al., 2013).

Evolution at the basis of the tree of life has been fascinating scientists for a long time(e.g., Margulis et al., 1990; Berney and Pawlowski, 2006; Parfrey et al., 2011). Reconstructing the phylogenetic tree that unites all lineages of eukaryotes is still a grand challenge (Hinchliff et al., 2015). The difficulty in defining homologous characters across the very different lineages makes it extremely difficult to resolve evolutionary processes and hence time scales. The incompleteness of consistent paleontological records of the delicate single-cell organisms which form the basis of the tree of life, makes calibration of evolutionary time scales imprecise. However, recent molecular biological studies provide a framework for a preliminary understanding of the timing of early eukaryote diversification, although estimated time scales differ greatly between the different investigations (e.g. Pawlowski et al., 1997; Berney and Pawlowski, 2006; Hinchliff et al., 2015).

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Until recently, several protistologists have assumed that single cell eukaryotes as microbial organisms should be potentially homogeneously and globally distributed due to supposed missing biogeographies and low geographical heterogeneity (Finlay, 2002). However, an increasing number of scientists lead by the ciliatologist Wilhelm Foissner (e.g. Foissner, 2006, 2007) has questioned the ubiquity model and proposed a so-called moderate endemicity model (e.g., Foissner, 2007; Weisse, 2008). Recently, studies based on next-generation sequencing support this idea: protists are extremely heterogeneously distributed in most aquatic and terrestrial habitats, even with local heterogeneities (de Vargas et al., 2015; Geisen et al., 2015).

However, it is unclear which processes have led to this enormous biodiversity. Investigating Earth evolution at the dry limit offers a unique research area to shed light on evolutionary processes and molecular substitution rates of protists. The extreme and distinct environmental conditions select for specific species molecular adaptations, supported by the well-defined fragmentation of the communities and populations by separation due to landscape and climate evolution in isolated populations (e.g. Gillespie and Roderick, 2014). Climatic thresholds that allow for a species migration may be different on species level. Unique to the research area is the fact that arid to hyperarid conditions in concert with high UV radiation create almost sterile conditions, especially in the core region of the Atacama (e.g., Azua-Bustos et al., 2012; Neilson et al., 2012; Valdivia-Silva et al., 2012), and spatially separate populations for different periods of time. The (hyper-) aridity of the Atacama Desert is mainly due to its geographic position in the subtropical high-pressure belt with its stable climate since the Late Jurassic (Hartley et al., 2005), the rain-shadow effect regarding Atlantic moisture transport established with the uplift of the Andes (Houston and Hartley, 2003), and to the restriction of humid onshore winds with the establishment of the cold Humboldt current in the Pacific (Rundel et al., 1991). Testing the mutual evolutionary relationship between biological evolution and geological (earth surface processes) processes, we aim to characterize thresholds for biological colonization (Fig. 1). In achieving these goals, we foresee major contributions to emerging concepts of evolutionary lag time (e.g. Guerrero et al., 2013), the interplay between geographical barriers and species migration in response to climate change (e.g. Burrows et al., 2014), species diversification in response to climate and geological processes (e.g. Gillespie and Roderick, 2014), and biogeomorphology (e.g. Corenblit et al., 2011).

1.2. Evolution of aridity in the Atacama Desert

The onset of aridity in the Atacama Desert is matter of an ongoing debate (e.g. Dunai et al., 2005; Evenstar et al., 2017; Ritter et al., 2018a; Hartley and Chong, 2002; Alpers and Brimhall, 1988). However, hyper-aridity is considered to be characteristic for parts of the Atacama Desert (between 21°–19°S in the Coastal Cordillera and partly Central Depression) since Miocene times (or even earlier Oligocene/Eocene, e.g. Dunai et al., 2005; Ritter et al., 2018a, 2018b; Evenstar et al., 2017). Subsequent expansion of arid conditions and intensification towards hyperaridity progressively expands from this core in all directions (Ritter et al., 2018a, 2018b). Therefore, hyperarid conditions in the Andean foothills to the east post-date the Miocene onset in the driest parts (Hartley and Chong, 2002). Long-term predominant arid to hyperarid climate was frequently punctuated by 'wetter' periods (still arid), that largely coincide with global climate events (e.g. Zachos et al., 2001; Jordan et al., 2014; Evenstar et al., 2017; Ritter et al., 2018).

1.3. Topography and distribution limits for organisms

Topography and climate gradients in the Atacama Desert may significantly control the recent distribution of organisms (Picard et al., 2008). Its extension is restricted by the Pacific Ocean to the west, and the rising Andes to the east, and covers the area from southern Peru in the north to the area around La Serena (Chile) in the south. Within this study, we will focus on the northern Atacama Desert, adumbrate by the 150 mm/yr precipitation isohyet (Fig. 2; Houston, 2006). Topography separates different habitats and strongly reflects climate conditions ('aridity'). The Coastal Cordillera acts like a barrier for most of the drainages ('migration corridors') sourced in the Andes, resulting in a more or less endorheic system of the Central Depression. Coastal areas experience frequent moisture supplies by coastal fog ('camanchaca', Stoertz and Ericksen, 1974; Goudie et al., 2002; Cereceda et al., 2008) creating fog oases (Pinto et al., 2006; Latorre et al., 2011). Between 23°–19°S hyperarid conditions prevail in most parts of the Coastal Cordillera and in the Central Depression, where biological life is strongly dependent on locally adapted organisms or restricted to perennial or ephemeral drainages fed by groundwater from the Andes. In this hostile environment, photosynthetic activity and primary production are almost at its dry limit (Warren-Rhodes et al., 2006). To the east, the rising Andean foothills towards the Precordillera experience increase precipitation and indicate a stepwise increase in biological colonization. The corresponding runoffs from the Andean foreslope enable the existence of riparian biomes (Quade et al., 2008; Latorre et al., 2013). Pre-Andean depressions, such as the Salar de Atacama, are intramontane basins enclosed between the Precordillera and the Western Cordillera, which, with regard to the Salar de Atacama, exhibit a long history as depo-center for clastic and evaporitic sediments of Cenozoic age (e.g. Jordan et al., 2007). The Western Cordillera and western parts of the Altiplano Plateau (> 4000 m a.s.l.) exhibit numerous endorheic basins, occupied by saline lakes and/or salt crusts, which are often delineated and created by volcanic activity and ignimbrite deposition. The adjacent Salar de Uyuni and corresponding other basins and salars, represent the main intramontane basin in the Altiplano, which repeatedly experienced lacustrine conditions in the past (e.g. Fritz et al., 2007, 2012; Baker et al., 2001, Baker and Fritz, 2015). Most of the high Andean basins receive their major precipitation from Atlantic sources

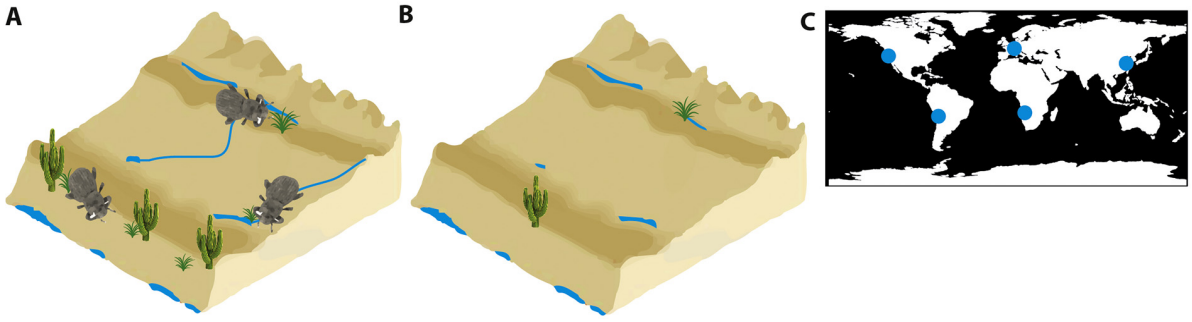

Fig. 1. Conceptual framework of the studies to resolve evolutionary processes in protists. Short-term rainy periods (50–100 yr) create corridors for distribution of plants and insects (A) while long-term dry conditions (2–3 Mya) in Atacama have led to separation from other similar regions (B & C).

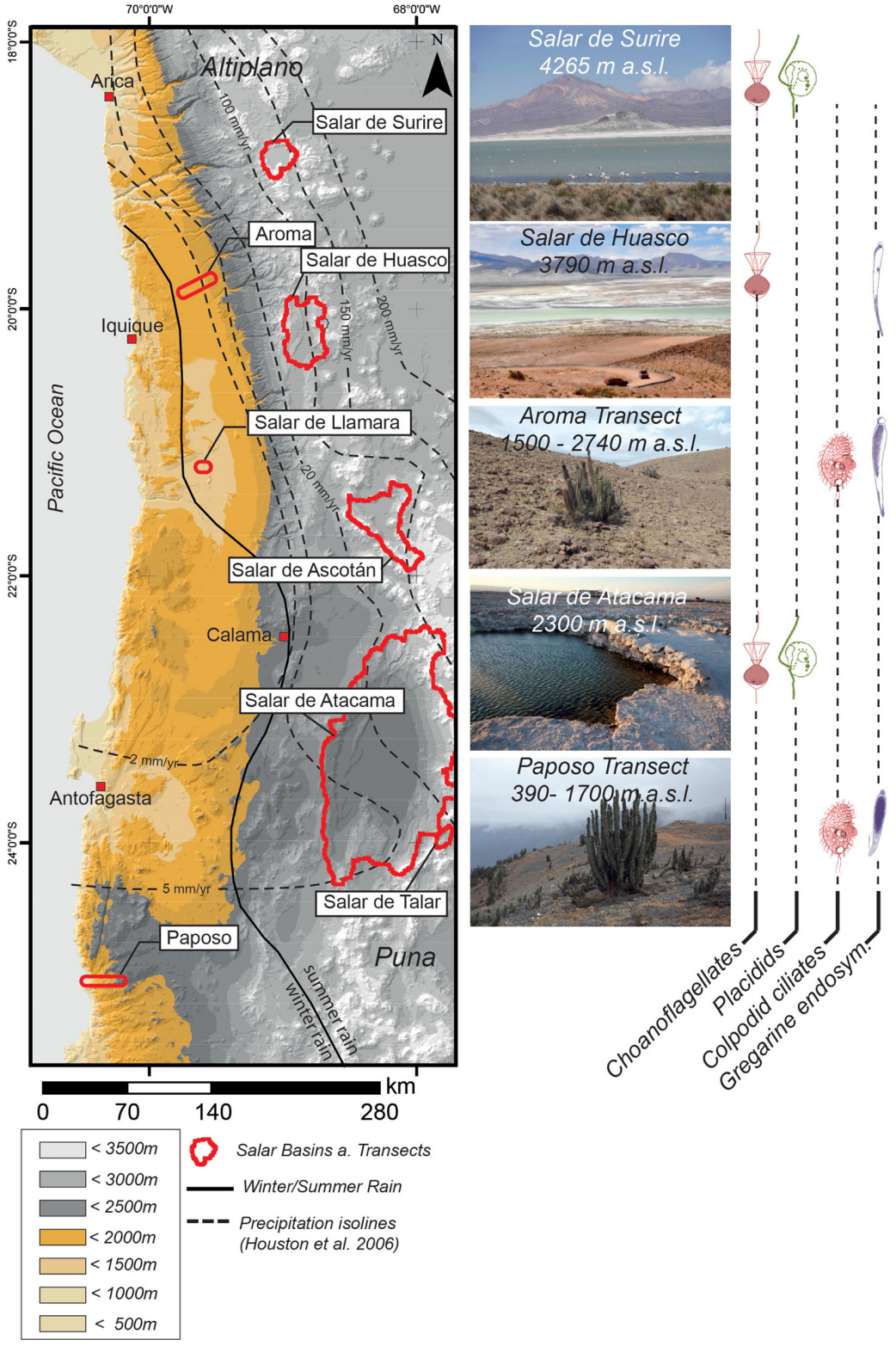

Fig. 2. Left: Colour shaded digital elevation model (derived from SRTM-data, created using ArcGIS 10.5.1) with isohyets (dashed black lines, Houston, 2006). Black line indicates the border between summer- and winter-rain dominated areas. Sampling sites are marked in red. Right: Representatives of four protists groups investigated exemplarily in this study and the local origin of investigated isolates. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and are connected to large drainage catchments (Altiplano drainage catchment including Salar de Uyuni, Coipasa, Lake Poopo; e.g. Placzek et al., 2011).

1.4. Extreme environmental conditions and habitat isolation as potential drivers of biotaxa evolution

Only a few plants, animals, and microbes managed to adapt to the extremely harsh environments of the Atacama region. Biologists were puzzled by the question of how living organisms could have adapted to survive in such a dry desert (e.g. Navarro-Gonzalez et al., 2003; Azua-Bustos et al., 2012). The patchy distribution of flora, fauna and microbiota in the Atacama Desert is assumed to be a result of contemporary environmental factors and historical contingencies. Landscape evolution should have influenced dispersal and thus the evolution and diversification of organisms, likewise climatic conditions and nutrient availability should have driven and should still drive dispersal and isolation processes of biological populations. The target areas are arid to hyper-arid systems, where the availability of water severely and predominantly limits both biota and Earth surface processes. In addition, the content of organic carbon in the soil as an energy source for heterotrophic life was estimated to be extremely low (Fletcher et al., 2012; Valdivia-Silva et al., 2012; Mörchen et al., 2019; Guerrero et al., 2013). Very high solar UV irradiance and the high concentrations of salts in the soil (Voigt et al., 2020) are additional factors limiting even microbial life in the Atacama Desert (Wierzchos et al., 2012; Mörchen et al., 2019)

The harsh environment in the Atacama forms one of the planet's most demanding conditions. This is reflected by the strong selection pressures for especially adapted organisms which have led to only a few evolutionary lineages coexisting under the extreme conditions. The strong fragmentation of plant populations (Böhnert et al., 2019; Merklinger et al., 2020) necessarily causes a strong fragmentation also of associated heterotrophic eukaryote populations e.g. in their phyllosphere or rhizosphere. The dominating insects are darkling beetles (Tenebrionidae) which are wingless and show a low passive dispersal (e.g., Cepeda-Pizarro et al., 2005). These insects are able to obtain water by digestion of dry organic matter or may collect moisture from fog (Hamilton and Seely, 1976; Willmer et al., 2000). Darkling beetles are known to harbour a diversity of endobiotic protists (e.g. gregarines), in many cases these associations of protists are known to be highly species specific (Desportes and Schrével, 2013) making them predestined model organisms for studies of co-evolution and associating molecular clock calibration for these protists (e.g. Ricklefs and Outlaw, 2010). It is believed that an extremely limited eukaryotic diversity mirrors the organic carbon restriction in these soil habitats. In contrast, aquatic protist communities in the very small ponds of the salars (highly hypersaline inland waters have been created by evaporating runoffs from Andean mountains) in the Atacama show a high number of unique protists, mostly not yet discovered and described (Triadó-Margarit and Casamayor, 2013). Diverse protists communities seem to be supported by diverse and productive prokaryote communities (Dorador et al., 2010).

As one hypothesis of our study it was assumed that plant radiation is younger than the onset of relatively constant hyperaridity (Picard et al., 2008; Böhnert et al., 2019) and that associated eukaryotic microbe communities show a relatively low divergence (if any) from related plant communities. Second, for several beetles, divergence has occurred already in the Atacama Desert (Zúñiga-Reinoso et al., 2019). Thus, those protists living as specific parasites/endobionts in beetles, which themselves may have been co-evolved with endemic plant communities, are assumed to show already a detectable divergence. Third, eukaryotic microbes living in salars may have adapted/co-evolved to the extreme environments independently and may reflect the extremely long stable geological conditions and geological isolation of the investigated water bodies in the Atacama Desert (Fig. 1).

The climatic and geological conditions have fragmented the area into habitats separated by nearly sterile regions without any eukaryotic life (Neilson et al., 2012; Valdivia-Silva et al., 2012). The habitable patches are several regions which allow a certain organic life due to specific adaptations to desert life or access to fragmented groundwater flow. In addition, short-term 'wetter' periods in the past could have enabled the opening (re-opening) and creation of biological migration corridors. We hypothesize that strong selection pressure may have led to only a few evolutionary lineages coexisting under these harsh conditions. To investigate our hypothesis, we combined studies on isolation, cultivation, morphology, ultrastructure, autecology, multi-gene phylogeny and next-generation sequencing on protist communities associated to the rhizosphere and phyllosphere of endemic plants (*Eulychnia* cacti), endemic species of darkling beetles (genera *Scotobius* and *Psectrascelis*), and on protists from hypersaline waters (salars). In the course of our studies, we intended to obtain different proxies to understand evolutionary processes at the dry limit due to interactions between evolutionary processes in the geo- and biosphere.

2. Material and methods

2.1. Study sites

Sampling was carried out during field trips in March 2015, 2017 and 2018 in the Atacama region of Northern Chile (Fig. 2). Samples were taken from hypersaline inland lakes, cacti phyllosphere and the gut of darkling beetles. The salinity of the original water samples taken from salars ranged between 6 and 310 PSU. Aquatic sampling sites included salars in the core of the Atacama (Salar de Llamará), in the Altiplano (Salar de Atacama, Salar de Huasco, Salar de Surire, Salar de Ascotan and Salar de Talar). Cacti phyllosphere was sampled in the Coastal Cordillera (*Eulychnia*; at Paposo) and in the Precordillera (*Browningia*; Quebrada Aroma). Gregarine endobionts were obtained from the gut of the darkling beetles *Scotobius brevipes* (at Paposo), *Scotobius* sp. (at Quebrada Aroma) and *Psectrascelis intricaticollis ovata* in the Altiplano close to Salar de Huasco (Fig. 3; details of sampling, preparations and sequences are given in Schiwitza et al., 2018;Nitsche et al., n.d.).

2.2. Isolation and cultivation

Protists (choanoflagellates and placidids) were isolated from salars and monoclonal strains were established either via the liquid-aliquot-method or single-cell manipulation and cultivated in artificial seawater medium (choanoflagellates: AB Reef Salt, Aqua Medic, Bissendorf, Germany; placidids: 35.256 g NaCl, 0.886 g KCl, 4.43 g $MgCl_2{\cdot}6H_2O$, 0.21 g $MgSO_4{\cdot}7H_2O$, 0.151 g $CaCl_2{\cdot}2H_2O$) with corresponding salinities. Isolated ciliate strains were cultivated in the WC media (Guillard and Lorenzen, 1972).

2.3. DNA sequencing and analysis (ciliates, gregarines)

2.3.1. Ciliates

DNA was extracted using the Quick DNA Microprep Plus Kit (Zymo Research, Irvine, USA) according to the manufactures instruction. SSU rDNA was amplified using the primer set 18sFor and 18sRev and PCR Mastermix (2×) (VWR Life Science, Red Taq DNA Polymerase, Hassrode, Belgium) with the following PCR program, 97 °C denaturation for 2 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 50 °C for 30 s, elongation at 72 °C for 2 min followed by a final elongation at 72 °C for 7 min. Positive PCR product were cleand up using the PCR Purification Kit (Bioscience, Jena, Germany) and Sanger sequenced using the primer 18sFor, 590For, 1300R and 18sRev. MegaX (Kumar et al., 2018) was used to calculate a phylogenetic tree using GTR-I-G (determined by MrAIC (Nylander, 2004)) as method for maximum likelihood analysis as basis for divergence time calculation.

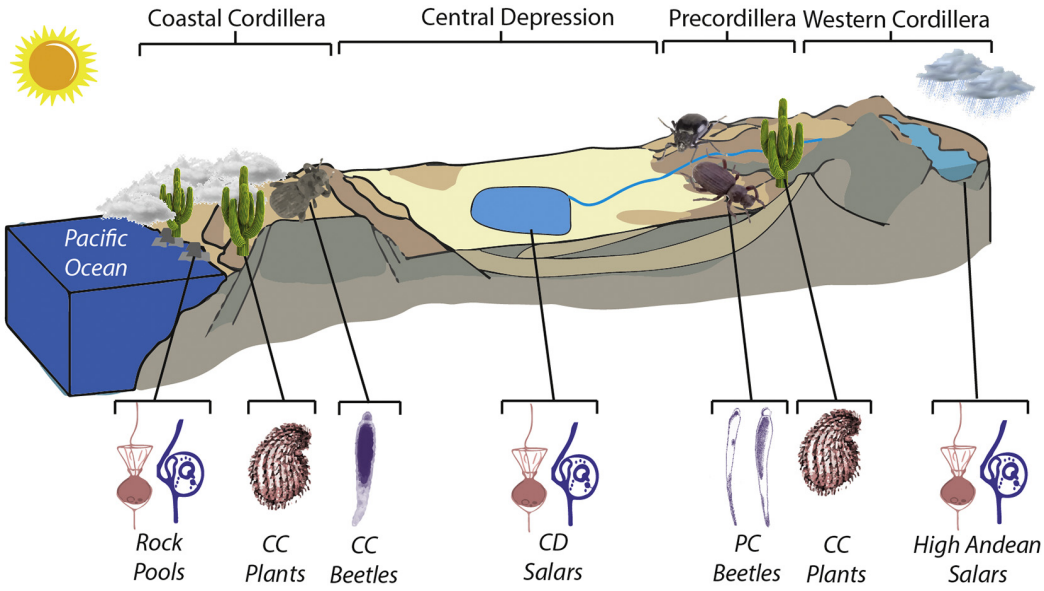

Fig. 3. Sketch profile through the Atacama Desert (modified after Pérez-Fodich et al., 2014) indicating different habitats of selected protist groups investigated in this study. Coastal areas experience moisture income through coastal fog, enabling the existence of fog oases, representing an isolated habitat of plants and insects. The Central Depression is dominated by arid- to hyperarid conditions, moisture income is largely controlled by connected high Andean drainage systems via groundwater or perennial flowing rivers (e.g. Rio Loa). Groundwater dependent populations are isolated within the Central Depression, however, may experience 'wetter' (still arid) episodes which enable a potential connectivity to other populations via temporal and spatial restricted migration corridors. Pre- and Western Cordilleran systems exhibit a higher direct moisture income and potential experience manifold genetic exchanges with other populations (CC = Costal Cordillera, CD = Central Depression, PC = Precordillera).

Molecular clock calibration was done after Wright and Lynn (1997), estimating divergence of the genera Scuticociliatida and Hymenostomatida at 1476–1640 Ma, *Coleps* and *Prorodon* at 709–784 Ma, *Bursaria* and *Platyophrya* at 835–928 Ma and *Bresslaua* and *Colpoda* at 158–176 Ma.

2.3.2. Gregarines

Ten trophozoites of each of the three species isolated from the gut of the tenebrionid beetles *Psectrascelis* sp., and *Scotobius* sp. and *Scotobius brevipes* were placed in a 1.5 ml Eppendorf tube containing 1 µl Proteinase K (AppliChem GmbH, Darmstadt, Germany). Lysis was carried out overnight. SSU rDNA sequences from the gregarines were amplified in one fragment using the self-designed primer Greg F (5′-CTGCGAAYRGCTCATANAAC) and a universal eukaryotic reverse primer, Rev -1 (5′-ACCTACGGAAACCTTGTTACG) in a 50 µl PCR with Taq 2× Master Mix. All amplifications consisted of an initial denaturation period (98 °C for 2 min; 35 cycles of 98 °C for 30 s), annealing period (48 °C for 45 s), extension period (72 °C for 2 min.), and a final extension period (72 °C for 10 min.). Subsequently, internal primers 82F (5′-GAAACTGCGAATGGCTC) and 1630 Rev. (5′-CGACGGGCGGTGTGTAC) were used together in a nested PCR with the following thermocycler program: initial denaturation (98 °C for 2 min; 35 cycles of 98 °C for 30 s), two step annealing (48 °C + 52 °C for 45 s, respectively), extension (72 °C for 60 s) and a final extension (72 °C for 10 min.). Selected PCR-products were cleaned-up by using the "PCR-Purification-Kit" (Jena-Bioscience, Jena, Germany). Purified samples were directly sequenced (GATC Biotech AG, Konstanz, Germany). The SSU rDNA sequences were initially identified by BLAST analysis prior to phylogenetic analyses. Divergence times were estimated using the Isthmus of Panama as a proxy, setting the separation of *Xiphocephalus triplogemmatus* and *Atacamagregarina* sp. Paposo at 6–20 Ma (Bacon et al., 2015). Calculations were performed using the ML analysis described above, applying the calibration points according to Parfrey et al., 2011 using the time tree function of MegaX (Kumar et al., 2018).

2.4. RNA extraction and transcriptome assembly (choanoflagellates, placidids)

RNA extraction and transcriptome assembly of choanoflagellate and placidid species was done according to Schiwitza et al. (2019). In brief, dense cultures were concentrated and RNA isolated using the peqGOLD Total RNA Kit (peqlab (VWR), Erlangen, Germany) following the manufactures instruction. Reads were assembled using Trinity 2.0.6 (Grabherr et al., 2011). Contigs were controlled using blastx against a reference database containing 15 proteins used for the analysis (Table 1). Amino acid sequences were arranged in the given order using BioEdit 7.2.1 (Hall, 1999). The suitable model for the maximum likelihood analysis was determined by MegaX 10.0.5 (Kumar et al., 2018), ML tree was calculated using the same program for of 5459 positions, the resulting tree was used for the molecular clock analysis. Time calibration was done according to Parfrey et al. (2011). For

Table 1
Fifteen proteins (and their abbrevation) used for the phylogenetic analysis and the calibration of the molecular clock for choanoflagellates and placidids.

Protein	Abbrevation
14-3-3 protein	14-3-3
40S ribosomal protein S8	40s
Actin	act
alpha-1 tubulin	αtub
beta-1 tubulin	βtub
Translation elongation factor 1α	EF1
Translation elongation factor 2	EF2
Enolase	enol
60S ribosomal protein L10	Grrc5
Heat shock protein 70	HSP70
Heat shock protein 90	HSP90
S-adenosylmethionine synthase	metK
Ribosomal protein S15A	Rps22
Ribosomal protein S23	Rps23
Transport protein Sec61	Tsec61

choanoflagellate species, an additional time knot was introduced, *Salpingoeca crinita* and *S. huasca* were restricted to 18–20 Ma, taking the higher mutation rates into account and the geological age of the Salar de Atacama. For choanoflagellates, 26 sequences were analyzed using the JTT-I-G model, using all sites, setting time calibration as follows: *Gallus gallus Homo sapiens* 328.3–400 Ma, *Schizosaccharomyces pombe/ Phanerochaete chrysosporium* 400–1.000My, *Apis mellifera/Drosophila melanogaster* 284.4–350 Ma, *Branchiostoma floridae/Homo sapiens* 520–555 Ma, *Nematostella vectensis/Capitella capitata* 632–800 Ma and *Branchiostoma floridae/Capitella capitata* 555–630 Ma. For placidis the LG-G-I-F was determined for of 5455 positions, time calibration was set as follows: *Phaeodactylum tricornutum/Thalassiosira pseudonana* 80–110 Ma and *Aureococcus anophagefferens/ Thalassiosira pseudonana* 133.9–550 Ma. Calculations were performed using the ML analysis described above applying the time nodes using the time tree function of MegaX 10.0.5 (Kumar et al., 2018). All accession numbers are given in Supplementary Table S1.

3. Results

Overall, the studies revealed the presence of a large variety of protists from all major phylogenetic groups of protists. In hypersaline athalassic waters, stramenopiles from various groups (placidids, bicosoecids, chrysomonads) dominated regarding their abundance followed by ciliates and different groups of heteroloboseans, but also choanoflagellates were found to be present in all samples. The phyllosphere of cacti was mainly populated by colpodid ciliates, cercozoans, heteroloboseans, kinetoplastids and amoebozoans among many other groups of protists. The gut of darkling beetles was populated predominantly by one group of apicomplexan protists, gregarines, up to 150 specimens were found in the gut of one beetle. The major gregarine species observed in the three different species of darkling beetles were morphologically and molecularly different from each other. For the purpose of the present study, we selected four protist groups as model groups for a deeper analysis of the molecular divergence within the group in the course of colonizing the Atacama region.

3.1. Craspedid choanoflagellates

In this study we present results on three of craspedid strains, which were found to be very different, considering in particular the rDNA, from all other known choanoflagellates (Fig. 4 A and Supplementary Material), clustering on a separated branch in the phylogenetic analysis (both, protein and DNA analysis; see Schiwitza et al., 2019), based on transcriptome data. The next related species is *Salpingoeca rosetta* isolated from North America. All three isolates from the Atacama belonged to different though related species (*Salpingoeca huasca, S. surira* and *S. crinita*). Divergence time estimates, based on relaxed molecular clock modus, infers major radiation of populations of the Atacama clade around 46.4 (9.2–5.4) Ma, and subsequent diversifications around ~20 Ma (19.1–21.9; *Salpingoeca crinita*). Radiation into Salar de Surire (*Salpingoeca surira*) and Salar de Huasco (*Salpingoeca huasca*) is inferred to have happened around ~2 (1.8 ± 0.2) Ma ago.

3.2. Placidid Stramenopiles

We found placidids in all hypersaline sampling sites (> 36 PSU) including rock pools in Pisagua. Based on transcriptome data, we were able to assign 8 different genotypes using 15 proteins for phylogenetic analysis (Table 1). A clear separation of evolutionary lineages from marine and athalassic waters (Fig. 4 B) is obvious, which dates back more than 114 (66–175) Ma ago. The estimated divergence times of the studied placidids from marine forms dates back to the opening of the southern Atlantic, break up of Gondwana and the separation of the South American continent (Torsvik et al., 2009; Fromm et al., 2015). At least three different genotypes could be isolated from Salar de Atacama, along with two significantly different genotypes from Salar de Llamara and also two different from Salar de Surire. This indicates some kind of radiation. Synchronous diversification compared to choanoflagellates occurred around ~18 Ma (12.0–29.1) and ~ 2.0 Ma. Consecutively, separation of genotypes from Salar de Atacama and Salar de Surire is inferred from strict molecular clock estimates to ~0.74 (0.33–1.76) Ma. The youngest separation seems to date back only about ~60 Kyr. The Salar de Atacama seems to have faced a multiple invasion of placidid species, about 5.8 and ~ 2 Ma ago. Interestingly, placidids strains from Poland, Germany and Canada are found in all three clades, separated from the Chilean species by low genetic distances.

3.3. Colpodid ciliates

The molecular identity of the different analyzed strains point to the fact that the species *Colpoda steinii*, *C. maupasi* and *C. inflata* analyzed here as model species for terrestrial ciliates, all showed large molecular diversity with p-distances of 0.4–6.7% within one “species” (p-distances among *C. steinii* 0.6–1.7%, among *C. inflata* 0.4–2.2%, among *C. maupasi* 6.7%; Fig. 5 and Supplementary Material). They have clearly to be considered as species complexes rather than species. We found completely different patterns of evolutionary processes. The investigated colpodids showed both, a high variability at the level of the SSU rDNA and also barley any differences. There is no clear signal of spatial or temporal separation of genotypes which could originate from habitat changes in the course of geological events. However, it is evident that even in this genus, which can be easily distributed by aeolian transport (e.g. Rivera et al., 1992), there are significant evolutionary distances between members of the same species complex. The *Colpoda inflata* – species complex showed a first invasion of the Atacama region max. 33 Ma ago followed by several divergence and invasion events between max. 11 Ma and present. However, all estimates of divergence times of colpodids are very uncertain (Fig. 5). The same pattern was recorded for the *C. steinii* – species complex (max. ~51 Ma, ~15 Ma, ~3 Ma), and a similar pattern also for the *C. maupasi* – species complex (max. ~40 Ma, ~17 Ma, ~10 Ma).

3.4. Apicomplexan gregarines

All three different investigated darkling beetle species (Pimeliininae) from the Atacama region in Northern Chile harbored different gregarines in their midguts. The gregarines (Apicomplexa: Eugregarinoridea, Stylocephalidae) *Atacamagregarina paposa*, *Atacamagregarina.* sp. 1 (Paposo), and *Atacamagregarina. spectrascelii* (Huasco) cluster with gregarines inhabiting other terrestrial invertebrates and form a separate clade with gregarines living in association with North American darkling beetles (Fig. 6). The evolutionary divergence of the three investigated gregarine species dates back to about 13 Ma (6–20). The three gregarine species have potentially evolved within 4 Myr in the different tenebrionid beetle species (*Psectrascelis intricaticollis*, *Psectrascelis* sp. and *Scotobius brevipes*) in the Atacama region (9.9 (3.9–17.5) Ma; 8.7 (3.5–15.1) Ma). Much later tenebrionids might have populated North America and coexisting gregarines might have coevolved with the following 6 Ma.

4. Discussion

4.1. General considerations

All groups of protists analyzed in this study and originating revealed a high degree of diversity in habitats of the Atacama region separated only by a few hundred kilometers. The lack of closely related sequences, in particular for choanoflagellates and placidids, from major databases like NCBI, VAMPS, ARB, PR^2 or the Tara Ocean Project, supports a moderate endemicity hypothesis, stating that also unicellular organisms have biogeographies (Foissner, 2006), irrespective of their potential to

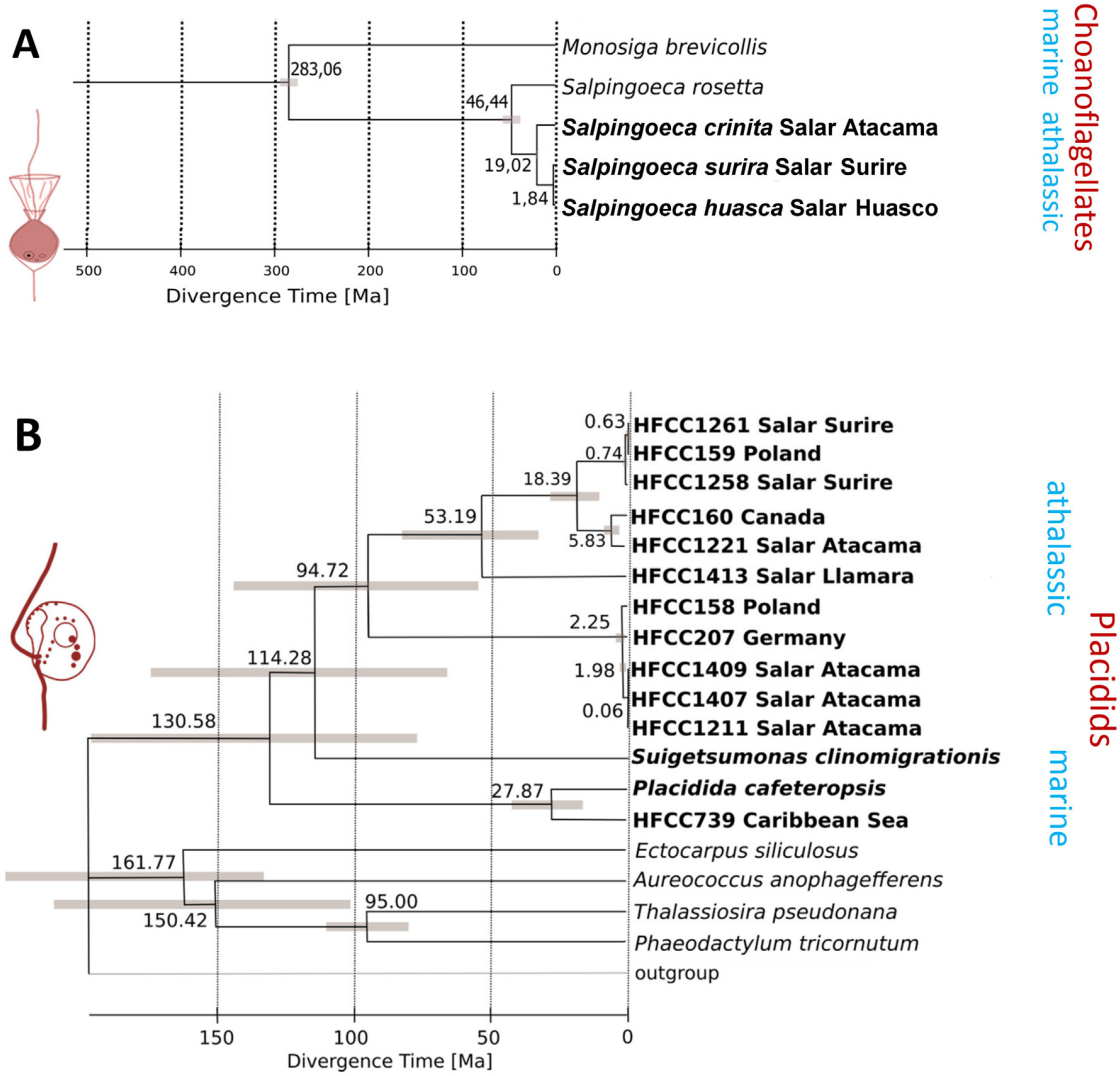

Fig. 4. Time-calibrated tree of choanoflagellates (A) and placidids (B). The time-calibrated tree of choanoflagellates is based on an analysis of 15 proteins and using calibration points regarding Opisthokonta (species indicated in the phylogenetic tree) of Parfrey et al. (2011) using a relaxed clock estimate. The time-calibrated tree of Placidida is based on an analysis of 15 proteins and using calibration derived for stramenopiles (*Ectocarpus siliculus, Aureococcus anophagefferens, Thalassiosira pseudonana, Phaeodactylum tricornutum*) from Parfrey et al. (2011) using a strict molecular clock estimate. Nodes are at mean divergence times and grey bars represent 95% HPD of node age. The x-axis show estimates of geological time scale in Ma. The tree of choanoflagellates is an enlargement of the whole analysis (see Supplementary Material) containing additionally the studied organisms and *Salpingoeca rosetta*. Divergence estimates are given in million years, rounded to two digits after the comma.

get distributed via air and other means than larger organisms. Still, undersampling can never be ruled out and must be considered to bias this assumption, but with the increasing amount of available data from NGS studies, we might get a much better resolution of protist distribution in future. Furthermore, the observed distribution patterns of genotypes and the analyzed number of mutations in each genotype compared to its most closely related genotypes in GenBank offers the chance to relate rates of evolution to the speed of geological and tectonic processes in the Atacama region.

Of course, estimates of evolutionary rates have to be treated with caution, since evolutionary rates are dependent on several factors. They are taxon specific and are dependent on different environmental parameters (e.g., Pawlowski et al., 1997; Parfrey et al., 2011). As the main aim of our studies was to compare evolutionary distances between protists of the same phylogenetic groups isolated from different habitats of the Atacama region rather than to estimate protistan evolution in general, we consider the potential bias introduced by taxonomic group specific differences in mutation and evolutionary rates to be acceptable (e.g., Berney and Pawlowski, 2006; Roger and Hug, 2006; Parfrey et al., 2011). When possible, we try to base our conclusions regarding the influence of geologic processes on independent evolutionary rates in different lineages. We considered that previous molecular clock studies yielded very different evolutionnary rates with a broad range of uncertainty (Parfrey et al., 2011). Using a relatively high protein and taxon sampling (15 proteins for placidids and choanoflagellates) from different phylogenetic lineages as well as – where applicable – the use of a relaxed clock estimate improves the chance to give a reasonable estimate of divergence times. These independent divergence estimates were then compared with estimates of divergence times of the habitats using geological and paleoclimatological archives.

4.2. Evolution of Atacama choanoflagellates

Our results indicate that the divergence of choanoflagellates has taken place within the Atacama region. The finding of three different choanoflagellate species, which form a distinct clade within the

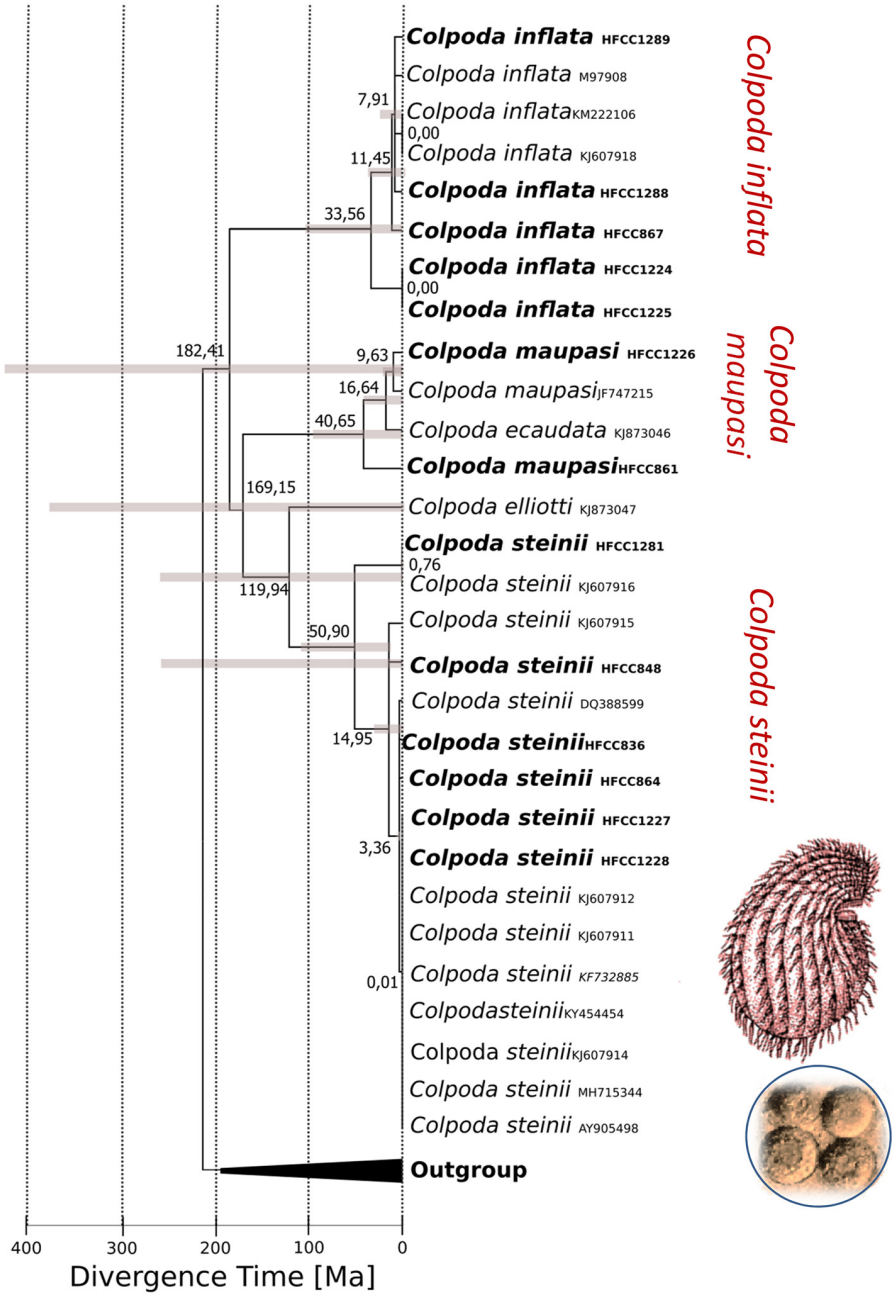

Fig. 5. Time-calibrated tree (18S rDNA) of colpodid ciliates isolated from Atacama samples (species names of Atacama isolates in bold) using the calibration points for colpodids from Wright and Lynn (1997) and applying a strict molecular clock of divergence times. Nodes are at mean divergence times and grey bars represent 95% HPD of node age. The x-axis show estimates of geological and absolute time scale in Ma. The tree of ciliates is an excerpt of the whole analysis (see Supplementary Material).

craspedid family distant from all other craspedid species discovered up to now, is a first indication of their divergence within the Atacama. The next related species, *S. rosetta*, recorded from Virginia, USA, was found to be euryoecious, i.e. tolerating freshwater to marine conditions, allowing to set up the hypothesis that this, or a closely related species, might have invaded the salars at an early stage, likely by migrating birds. Groundwater might be considered as another source for dispersal. But considering the flow direction of the groundwater feeding the salars, flowing from the Andes to the coast, a reinvasion of the elevated, younger salars is not very likely. Therefore, we favour the dispersal by birds or by Aeolian transport. According to the estimated divergence times of athalassic choanoflagellates from marine clades an invasion ~50 Ma ago from marine to the Atacama could already point to arid conditions in the Salar de Atacama at that time. Strongly intensified aridity around 20 Ma could have enabled the divergence of the Salar de Atacama and Surire/Huasco clade, which would set the divergence estimates of the individual Surire and Huasco species to have happened around ~2 Ma. The youngest divergence could potentially be connected to the isolation and closure of distribution corridors and cutoff of population-wide gene flow and exchange in the high altitude area, coinciding with the onset of the Quaternary (see Fig. 7).

As most of the inland lakes within the Atacama region are shallow water bodies with barely any turbidity, UV radiation is a severe problem for non-pigmented or otherwise protected aquatic organisms. We hypothesize that positive mutations driven by a high mutation rate caused by high UV radiation may be responsible for the divergence of

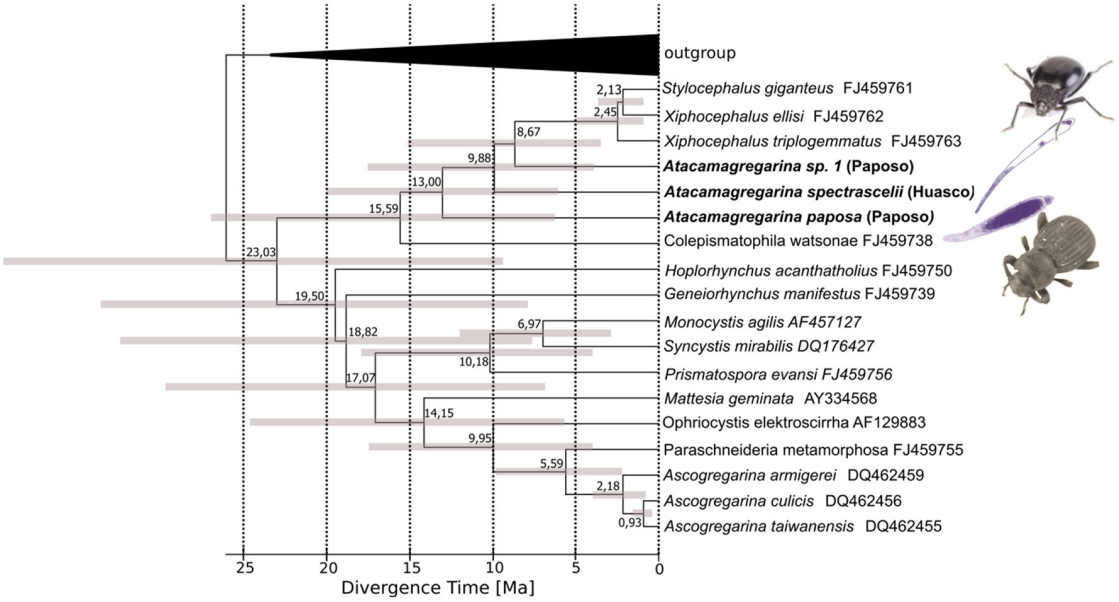

Fig. 6. Time-calibrated 18S rDNA-tree of gregarines inhabiting beetles from Atacama (*Atacamagregarina*) and from those available in GeneBank. The tree is based on calibration points for Alveolates by Parfrey et al. (2011). Node of divergence of *Karenia* and *Crypthecodinium* and of *Crypthecodinium* and *Heterocapsa/Alexandrium* were calibrated with Phanerozoic fossils (Parfrey et al., 2011). Nodes are at mean divergence times and grey bars represent 95% HPD of node age. The x-axis show estimates of geological and absolute time scale in Ma.

the different species. Assuming that the divergence of an athalassic choanoflagellate (*Salpingoeca crinita*) took place before about 20 Ma or even much earlier, when abiotic conditions in the Salar were likely comparable to brackish water, the rising abiotic stress through evaporation and rising ion concentrations in combination with high UV radiation may have favoured the divergence process. The autecologial experiments by Schiwitza et al. (2018) showed a very high salinity tolerance of the three discovered craspedid choanoflagellates, which point to the invasion of the species from coastal environments. Their ability to reproduce at low salinities (5–12 PSU) is an indication that an early invasion into the young salars may have taken place. As no related sequences have been recorded from anywhere else in the world (including several databases, see above), we hypothesize that a divergence has taken place within the Atacama region. Longer episodes with athalassic waters established in the Quillagua-Llamará basin and potentially adjacent basins (Sáez et al., 1999, 2012), which may have created long-lasting isolations of protest populations with high salinity tolerances, may have also favoured the evolution of Placidida diversity we have observed. This is also supported by the isolation and description of another very specific choanoflagellate from this region, an extraordinary athalassic acanthoecid choanoflagellate from the newly described genus *Enibas* (Schiwitza et al., 2019), indicating parallel evolution of this adaption to highly saline athalassic waters in choanoflagellates.

4.3. Evolution of Atacama placidids

The clade of athalassic placidids probably evolved from marine forms already 131 (77–196) Ma ago, long before the development of the Atacama region. Up to now there were only four species described from marine habitats, while in the Atacama alone, at least four new species could be identified by us, none of them was closely related to species found in coastal marine rock pools of the Pacific coast (unpub. data). The placidid protists seem to contain a clade of heterotrophic flagellates especially adapted to highly saline athalassic waters. The studied salars have to be considered as a center of radiation in the Atacama Desert for placidids due to its isolation from other desert regions, the distance between the different salars and the fragmentation of habitats within several salars (see above). This might have supported allopatric as well as sympatric divergence processes. This idea is supported by the coexistence of different species in one and the same salar system today. Divergence occurred around ~18 Ma with the divergence of a Salar de Atacama clade and a Surire/Huasco clade, which coincide with divergence estimates from choanoflagellates. Further divergence took place in the Salar de Atacama about 5 Ma coinciding with higher ignimbrite frequency (Salisbury et al., 2011; Evenstar et al., 2016). The Salar de Atacama, as the oldest isolated salar (Salisbury et al., 2011; Evenstar et al., 2016), seems to have faced multiple divergence processes and/or invasions of placidid species within the last about 6 My. The large genetic distances of the placidids originating from athalassic waters with regards to those from coastal waters may indicate the seldom chance of successful invasion and adaptation to athalassic conditions of divergently evolved populations from marine environments. Though there were exchanges between different populations worldwide (e.g. Atacama, Canada, Europe, all originating from hypersaline habitats, see Fig. 4B), these exchanges were limited to exchanges between different athalassic rather than between athalassic and marine waters. These exchanges might be due to aeolian transport of the small and light cysts of placidids. Since the area of athalassic waters is extremely small compared to soil, marine and freshwater habitats from which cysts could have been distributed by aeolian transport, the chance of exchanges between different athalassic populations worldwide should be extremely small. This is reflected by invasions/exchanges of genotypes dating back to about 0.6 Ma and more (Fig. 4B). This seldom chance of exchange may have supported the radiation of species in the different salars of the Atacama. The placidids in the Atacama region seem to be the first example until now, where complex radiation processes in a protozoan group are visible shedding light on the divergence processes in the past.

4.4. Evolution of Atacama colpodid ciliates

Colpodid ciliates are especially adapted to live in the phyllosphere and upper soil horizons due to its ability to encyst and excyst within minutes depending on the presence of water and to divide within cysts (Barker and Taylor, 1931). The genetic patterns observed for three

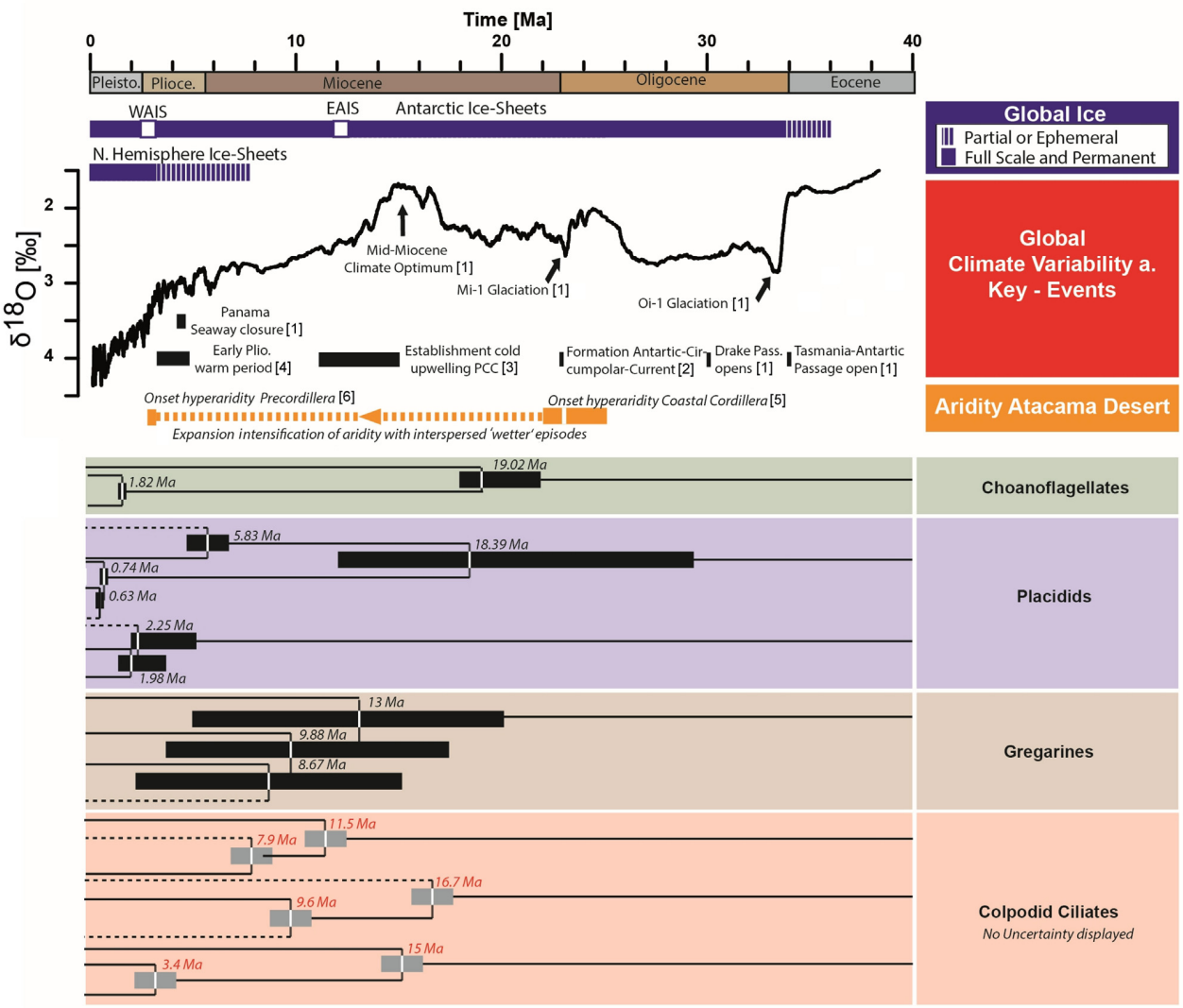

Fig. 7. Global deep-sea oxygen isotope stack based on data from Zachos et al. (2001). Vertical blue bars indicate a qualitative representation of ice volume in each hemisphere relative to the LGM, dashed parts indicate episodes of minimal ice cover (< 50%), full bars represent close to maximum ice coverage (> 50% of present) from Zachos et al. (2001). Major global tectonic and climatic periods/events are marked [2] Kennett, 1982; [1] Zachos et al., 2001; [3] Houston and Hartley, 2003; [4] Wara et al., 2005, as well as evolution of aridity in the Atacama Desert [5] Dunai et al., 2005, [6] Hartley and Chong, 2002. Synopsis of time estimates for phylogenetic divergences and radiations of protists in the Atacama (simplified, for details see Figs. 5–7). Dashed lineages indicate non-Atacama species, bold lineages represent Atacama protist groups. Uncertainties for divergence times of colpodid ciliates are not displayed due to the large uncertainty. For more explanation see text. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

investigated species complexes (*Colpoda steinii*, *C. maupasi* and *C. inflata*) were very similar. Representatives of the recorded colpodid species complexes are found worldwide in all kinds of terrestrial habitats (e.g. Bowers et al., 1998). However, the high number of coexisting genotypes we recorded at our few sampling sites in the Atacama region astonished us. This might indicate that the low number of competing ciliate species in this extreme environment, their isolation from other regions and their high adaptability to harsh arid and hyperarid conditions may have allowed for a relatively fast radiation supported by invasions from other regions. At just one sampling site at least five different genotypes were found coexisting. An increase in the number of sampling sites and the number of isolates per site would be necessary in future to get a better resolution of distribution patterns of genotypes.

Based on the high uncertainties within the molecular clock analysis, no robust assumption on the invasion times can be given, since calibration points were only available for ciliates distant to colpodids (Wright and Lynn, 1997). Our data indicates an invasion of the Atacama region by *Colpoda inflata*, *C. steinii* and *C. maupasi* long before significant Andean uplift and subsequent reduction of aeolian transport from the Atlantic. Younger invasion/divergence events (~17–11 Ma) based on divergence estimates coincide with the uplift of the Andes and evolution of the Altilplano Puna Plateau, which intensified (1) the rain-shadow effect causing the intensification of arid to hyperarid conditions on the Atacama side; (2) the reduction of potential aeolian transport; (3) a major re-organisation of plant species to new climatic conditions, including new habitats due to pristine unpopulated high altitude areas (Picard et al., 2008) and also the creation of a geographical barrier for migration of organisms over the Andes, such as plants (see also Böhnert et al., this issues). Younger divergence events most likely have followed specific divergence processes within the Atacama Desert due to the evolution towards hyper-aridity throughout the Atacama Desert (expansion of arid conditions towards eastern and higher elevated areas, Hartley and Chong, 2002; Evenstar et al., 2016; Jordan et al., 2014).

4.5. Evolution of endobiotic gregarines in Atacama beetles

The analysis of the evolutionary distances of the gregarines isolated from three different populations of darkling beetles supports the hypothesis of co-evolution of endobionts with their hosts. One gregarine/beetle population was collected from the Coastal Cordillera (*Atacamagregarina* sp. Paposo from *Scotobius brevipes*), while the other endobiont/host population was obtained from the Precordillera (*Atacamagregarina* sp. Huasco from *Psectrascelis intricaticollis ovata*) about 500 km apart connected with a north-south and east-west climatic gradient which influences the chance of beetle distribution along the Atacama Desert. Isolation of darkling beetles with their endosymbiotic gregarines (from ~13 Ma onwards) might be connected to the emergence and evolution of the high Andean plateau, causing the establishment of one of the major geographical barriers in South America for organisms (plants, insects, mammals etc.). This in concert with subsequent expansion and intensification of overall arid conditions in the Atacama Desert could have led to isolation and divergence of specially adapted darkling beetles and subsequent divergence of endosymbiotic gregarines. The evolutionary distances indicated that the three gregarine species which have evolved within the Atacama region within nearly 4 Myr are distant from those in other regions and host animals. It is probable that the closure of the Isthmus in Panama (Bacon et al., 2015) might have allowed darkling beetles from the Atacama region to migrate to North America leading to the divergence of beetles and co-evolution of their endobionts 2–3 Ma ago (*Stylocephalus* and *Xylocephalus*, Fig. 6). Further taxon sampling is necessary to understand the co-evolutionary processes better.

4.6. Evolution of aridity in the Atacama Desert as driving factor for biological diversification

The Andean orogeny, the evolution of the Atacama Desert with increasing aridity since the Oligocene/Eocene (e.g. Dunai et al., 2005; Evenstar et al., 2017; Ritter et al., 2018) and the global climate deterioration since the Eocene, enabled geographical isolation and radiation of biological species in South America, especially in the Atacama Desert. Recent compilations (e.g. Jordan et al., 2014; Evenstar et al., 2017; Ritter et al., 2018) of the evolution of climate and aridity in the Atacama Desert indicate that aridity has developed heterogeneously in time and space and cannot be generalized for the entire Atacama Desert. Intensification and expansion of aridity in the Atacama Desert proceeded gradually with time through the interaction of different geological (e.g. Andean Orogeny, tectonic opening of oceanic passages) and climatological events/processes (e.g. glaciations, Mid-Miocene Climate Optimum, Zachos et al., 2001). In order to identify potential forcing factors for biological radiation, derived molecular data can be compared to global Cenozoic climate trends recorded by $\delta^{18}O$ variations in marine sediment records and key tectonic events (Zachos et al., 2001), such as the opening of the Drake Passage, Closure of the Isthmus of Panama or the uplift of the Andes and the specific climate evolution of the Atacama Desert (Fig. 7).

The earliest invasion and evolution of athalassic protists (choanoflagellates, placidids) around ~50–40 Ma could indicate the existence of saline to hypersaline habitats in the Atacama Desert, e.g. Salar de Atacama. This could be partly supported by studies from the Salar de Atacama, which reveal the deposition of silt and salts of the San Pedro Fm (Pacienca Group, Jordan et al., 2007; Evenstar et al., 2016) during Late Eocene/Oligocene to the Early Miocene. Contemporaneous radiation of choanoflagellates and placidids around ~20 Ma could be interpreted as a region-wide signal with a common forcing mechanism. Studies from the low-lying Atacama Desert indicate the onset of hyperarid conditions in parts of the Coastal Cordillera and Central Depression with the beginning Miocene (e.g. Dunai et al., 2005; Evenstar et al., 2017; Ritter et al., 2018). A major hiatus in the Salar de Atacama around ~17 Ma (Jordan et al., 2007; Evenstar et al., 2016) could indicate more arid conditions in the salar, which probably minimized habitats. This intensification of arid conditions on the western side of the Andes coincided with progressive formation of the Antarctic Circumpolar Current (~23 Ma, Kennett, 1982; Zachos et al., 2001) and intensification of the cold Humboldt Current. Uplift reconstruction of the Western Cordillera additionally point to elevations above ~4000 m (Scott et al., 2018), intensifying the severity and onset of the Andean rain-shadow effect.

Later, more recent, divergence of athalassic protists (choanoflagellates, placidids), around ~2–3 Ma, might have led to a separation of species which had already been adapted to extreme environmental conditions (high salinity, aridity; we observed that some strains are able to survive salinity changes between 4 and 250‰ S, e.g. Schiwitza et al., 2018, 2019) (see Fig. 7). Intervening climate variability which caused divergence of other protist species to adapt to current arid climate conditions, could not be proved for choanoflagellates and placidids which may indicate that adaption to arid conditions of present organisms was sufficient enough to tolerate subsequent climate variability. The divergence estimates of around ~2–3 Ma where similar for several species of choanoflagellates and placidids indicating an region-wide cause of divergence, which most likely was connected to climate induced isolation of habitats and cutoff of genetic exchange due to closure of distribution corridors with the beginning Quaternary (global cooling) and intensification of arid conditions and expansion of hyperarid conditions into the Precordillera (Hartley and Chong, 2002). The global climate underwent a major cooling since the Early Pliocene warm period (~3–4 Ma), leading to the establishment of the permanent ice sheet at high latitudes and the establishment of the West-Antarctic Ice-Sheet (Zachos et al., 2001). Furthermore, climate models indicate that the reinforcement of the Humboldt Current, especially at the Plio-Pleistocene transition was the important factor for the establishment of widespread hyperarid conditions within the Atacama Desert (Garreaud et al., 2010). Global climate deterioration towards the Quaternary commenced establishing widespread hyperarid conditions in the entire Atacama Desert. Most recent divergence estimates of Placidids in the Salar de Surire coincide with a major change in the Quaternary during the Mid-Pleistocene Transition and the switch towards the 100kyr world, causing high amplitude climate oscillations (e.g. Hewitt, 1996; Zachos et al., 2001; Tziperman and Gildor, 2003; Médina-Elizalde and Lea, 2005; Clark et al., 2006).

Divergence and isolated divergence of darkling beetles and their endosymbionts (endobiotic gregarines) indicated a co-evolution. Mid-Miocene divergence estimates (~13–9 Ma) could be connected to the uplift of the Altiplano-Puna Plateau (e.g. Garzione et al., 2017), creating one of the major geographical barriers for migration of biological organisms in South America. Additionally, an enhanced rain-shadow effect of the rising broad Altiplano-Puna Plateau, favoured the intensification and expansion of arid conditions in the rain shadow regions to the west, i.e. the Atacama Desert. Isolation, adaption and divergence of darkling beetles and their endosymbiotic gregarines are tracked by subsequent divergence estimates of ~13 Ma, ~10 Ma and ~ 9 Ma. The absence of younger divergence estimates could be an indication, that populations were already sufficiently enough adapted to arid-hyperarid conditions to survive following shifts to even more extreme aridity/climate conditions. Similar diversification ages of plants, such as *Cristaria* (Böhnert et al., 2019) also show the split of a Mediterranean clade and Atacama clade during the Mid-Miocene which could be connected to increasing aridity.

Divergence estimates of colpodid ciliates can be partly interpreted, due to their ability to easily invade new habitats via aeolian transport, as invasion/colonization ages to specific regions. Earliest divergence estimate of ciliates around ~30–50 Ma point to the early colonization of the Atacama region. Subsequent simultaneous divergence estimates, i.e. colonization of two colpodid ciliate clades around 17–14 Ma, could be related to stable climate conditions coinciding with the Mid-Miocene Climate Optimum (Zachos et al., 2001). Younger divergence estimates

reveal similar ages like gregarines which may be connected to each other, and could have similar forcing factors, i.e. uplift of the Altiplano-Puna plateau and increasing aridity. Youngest divergence estimate around ~3 Ma could coincide to short stable conditions during the Early Pliocene warm period (Wara et al., 2005). The youngest divergence estimates from colpodid ciliates ~0.7 Ma can be related to high amplitude climate variability during the Quaternary causing isolation of habitats and the cessation of gene flow or genetic exchange.

4.7. Comparison to other divergence lineages in the Atacama Desert

Our estimates of divergence times were – depending on available data sets – derived from either a multigene (15 protein) analysis (choanoflagellates and placidids) or due to estimated mutation rates for the 18S rDNA gene (colpodids ciliates and gregarines). For colpodids, calibration points were only available for ciliates distant to colpodids (Wright and Lynn, 1997), for gregarines a relaxed molecular clock approach was applied assuming that North American populations could evolve only after the closure of the isthmus. For choanoflagellates and placidids we were able to use transcriptomics to get the 15 protein sequences for the non-model organisms to be able to use the method used by Parfrey et al. (2011) and make use of their calibration. In all cases the resulting divergence rates could be related to geological processes and the divergence rates were independent of the method relatively similar. Evolutionary rates were variable as one should expect for different phylogenetic lineages (e.g. Pawlowski et al., 1997), however, they were in the same range as published for protists (Parfrey et al., 2011; Sánchez-Baracaldo et al., 2017; Lutzoni et al., 2018). For the colpodid ciliates, the range of uncertainties was huge, however, for the other groups of protists, the obtained time scales were - regarding the level of uncertainty - comparable to the accuracy of geological time scale estimates (Picard et al., 2008; Böhnert et al., 2019). fthe target regions, an estimate of geological separation of the different catchment areas in the Andean should be possible. From our studies presented here, we can at least conclude that the catchment areas (see Fig. 2) were separated since more than ~2 Ma. Such combined dating using biological and geological/tectonic archives might help in future to further resolve the timing of geological/tectonic events and vice versa (e.g. Picard et al., 2008).

5. Conclusion

Our analysis of the diversity and divergence within several lineages of protists in different habitats of the Atacama revealed patterns fitting to the geological and climatic changes from the late Eocene to the onset of the Quaternary. Several coexisting species point to the repeated invasions, whereby some species already adapted to extreme environmental conditions. Multiple divergence/invasion events can be best explained by alternating climate variability favouring the re-opening and closure of migration corridors enabling extended gene flow and the coexistence of several species. In contrast to the invasion and colonization of highland plant taxa in the uplifted Andes (Hughes and Eastwood, 2006), the evolution of the Atacama Desert and increasing aridity led to the long-term reduction of habitats and as a consequence to a decrease in species, if no adaption to extreme conditions occurred.

The signal of the 'progressive uplift' of the Western Cordillera combined with an onset of aridity in the low-lying Atacama Desert (which means also intensified aridity in higher elevated areas) should have led to a strong segregation of migration corridors about 20 Ma ago and probably much earlier. The evolution of the choanoflagellate *Salpingoeca crinita* and an athalassic lineage of placidids might took place in this period. During the Miocene, aridity intensified and expanded towards the eastern parts of the Atacama and upslope the Precordillera and Western Cordillera. The migration pathways possible in that time due to periodic vegetation might have favoured divergence of darkling beetles and co-evolving endobionts. The onset of Quaternary and climate deterioration since ~2 Ma ago led to a further expansion of arid conditions in higher elevated areas and to a closure of migration pathways so that evolved species got further isolated and cycles of recurrent isolation and secondary contact of populations led to further divergence (e.g. choanoflagellates, placidids). The Mid Pleistocene transition (100 kyr cycle) initiated high climate variability with high amplitudes, which may again have favoured divergence processes of protists within the Atacama (colpodid ciliates, placidids).

Protist populations from salars within the Atacama which had relatively constant hyperaridic conditions for several 100 thousands years indicate radiation of at least two of the investigated groups of protists, choanoflagellates and stramenopile placidids. We assume that the large, literally sterile (considering protists) desert surrounding at the different salars may cause a low exchange rate of protists specifically adapted to athalassic high salinities. This might have been a trigger for divergence but also for genetic processes such as reticulate evolution.

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Author contributions

All authors were involved in the sampling and preparation of samples; the geological context and age constraints were provided by B.R. and T.D.; investigations on the cultivation and isolation of protists was mostly carried out by S.S., A.R., F.N., H.A.; molecular investigations were conducted by F.N. and morphological analyses were done by S.S., A.R., F.N., H.A.; molecular clock analyses were made by F.N.; H.A., B.R. and F.N. were involved in the conceptualization and writing of the original draft and supervised the studies. All authors reviewed and edited the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Conclusive Summary and Perspectives

Within this thesis, the main objective was to enlarge the knowledge about protists' diversity, adaptation, community composition and distribution in extreme environments, illustrated by the Atacama Desert in Chile. A particular focus was placed on hypersaline inland waters (salars), which require special adaptations for their inhabitants due to their extreme conditions, e.g., high and varying salinities, high temperatures, high UV radiation and the adaptation to high altitudes. Before our studies, only a few eukaryotic organisms had been reported from hypersaline water bodies, and the knowledge of heterotrophic protists was low. Investigations on protists should always combine the molecular data with morphological, ecological and behavioral characters (Boenigk et al., 2012). Within this study, 28 strains from Placididea (23 strains), Percolomonadida (2 strains) and Cafeteriaceae (3 strains) were isolated and cultivated, and the number of described taxa was enlarged in total by six new genera and 17 new species based on the combination of morphological, molecular, and ecological investigations. We also enlarged in this context the knowledge of taxa, isolated from extreme high salt concentrations (by two genera, eight species) and observed a high diversity within placidid species. Due to the large morphological similarities and the relatively high inter- and intraspecific variation within placidids, it was particularly important to investigate additional properties of their representatives. Investigations of strains from various globally distributed sites including hypersaline, marine and brackish waters from Chile, Germany, Kenya, Canada, Japan, the Atlantic Ocean, and the Caribbean Sea showed that placidids harbour an unexpected high species number, mainly from extreme habitats. We compared our isolated placidids to already described species and performed further investigations.

Transcriptomic data were added through phylogenetic analysis, and additional salinity tolerance experiments were performed to classify and describe these species as precisely as possible. The prior absence of placidid sequences in many databases could be explained on the one hand by their preference for a benthic lifestyle and, on the other hand, by their specific habitat requirements within their hypersaline environments. Placidids seem to be especially adapted to hypersaline environments and their changing salinities. But it is still unknown which exact mechanisms they use for this adaptation. Investigations of salinity tolerances of the various placidid species indicate that this ecological parameter might be an important driver for species separation. Future studies on the special adaptation would be particularly interesting. In many phylogenetic studies, the nuclear small subunit rDNA is widely used as a nuclear marker gene (Stoeck et al., 2010; Fonseca et al., 2014; Lie et al., 2014). It is highly conserved compared to other genes due to its slower evolution (e.g. mutation rate), and intraspecific variation could hardly be resolved (Parfrey et al., 2011; Arndt et al., 2020). The transcriptomic data of next-generation sequencing provided previously unavailable information of placidids, and a phylogenetical classification was possible based on multiple (variable and conserved) genes. In the past, multigene analyses have successfully resolved the phylogeny within several protist groups (Brown et al., 2013; Pánek et al., 2015; Carr et al., 2017; Lax et al., 2021), and the usage of more than one gene could greatly improve the resolution of the tree (Burki et al., 2020). In the present study, the isolation and description of species provided an idea about the great undescribed diversity of heterotrophic flagellates, especially in hypersaline environments. Metabarcoding analyzes also reinforced the assumption of the hidden diversity of placidids. The unfiltered data revealed many genotypes of placidids. However, it was not possible to say whether these organisms are present as cysts or as reproductive and adapted organisms based on DNA isolation. Under culture conditions, we observed the formation of these dormant stages with

rising salinity due to evaporation. Survival and dispersal would be possible at non-optimal conditions (Rogerson and Detwiler, 1999). Therefore, also the RNA should be isolated and analyzed from the systems in the future. The occurrence of genotypes within the salars that were originally only isolated from fresh, brackish or marine waters (e.g. *Suigetsumonas clinomigrationis*) should be therefore viewed with caution and verified by cultivation approaches in the future. This is true not only within the group of Placididea but also for all the different taxa. Especially in the Salar de Huasco and the Salar de Atacama with its several different groundwater inflows of different salt compositions and concentrations, it could be possible that representatives of these species could be flushed into the salar systems, formed resting stages due to rising salinity and remain there until the conditions change again. The occurrence of these organisms would still be registered in DNA studies. Nevertheless, the V9 region of 18S rDNA turned out to be a good tool to understand the diversity of the microbial community if the read number is taken into account (Choi and Park, 2020). Within our metabarcoding approach, we conducted two different filter methods and compared them to each other. By applying a relatively strong read filter, where a mock community was used as a reference community, we tried to significantly reduce sequencing errors and the overestimation of taxa richness. However, the disadvantage of this method is that due to the strong filtering, a possible overlap could not be found, although it would potentially be there. The filter may seem a bit strong because a lot of information gets lost, but in comparison to the approach used by others (e.g. Schoenle et al., 2021) where the threshold of, e.g. three reads was applied, we see nearly the same relative distribution and come to the same conclusion in community composition. The strong filter allows us to make a better statement about potentially present organisms. Various genotypes could not be assigned to any taxon, and a hidden diversity was still visible. Further sampling and cultivation approaches and ecological investigations should be conducted to get more

information about the protistan representatives inhabiting these environments and which have been overlooked or could not be isolated so far. Based on the metabarcoding analyses, we were able to compare and make initial statements about the community composition at 24 different aquatic habitats in northern Chile and got an idea regarding the overlap or separation of the unicellular eukaryote communities. Further, we gained initial insights into the possible transport, distribution, and exchange of protists between different habitats. Each extreme aquatic system showed its unique protist community and species composition also, within the different microhabitats at one salar is very diverse and overlaps only a little. This suggests that the salars might be considered as separated systems with only low possible exchange. The organisms seem to be restricted in their distribution between different salars and already within one salar. These systems seem to be places where allopatric speciation might have taken place, assuming that the rate of evolution is higher due to the high reproduction rate than their rate of dispersal and adaptation to the new habitat. Representatives of Stramenopiles (Ochrophyta and non-Ochrophyta) and Alveolata (Ciliophora) dominated all samples and seemed to be particularly adapted to these extreme environments. Within the metabarcoding approach, we also tried to make first statements about the potential occurrence and distribution of placidids. Currently, there are only two species within the class of Placididea containing the same V9 region of 18S rDNA. The other representatives showed relatively high variability between species (up to 21.5 %). Thus, it seems that placidids could possibly be distinguished down to the level of genus based on the V9 region of 18S rDNA. Especially, representatives of the genus *Allegra* were able to cope with an extremely high range of salinities (20-284 PSU). *Allegra hypersalina,* e.g., could primarily only be isolated from the Salar de Llamará so far. However, the genotype of *A. hypersalina* was detected by metabarcoding also in four different salars (Salar de Llamará, Salar de Huasco, Salar de Atacama, Salar de Coposa), the groundwater inflow into the

Salar de Llamará, one geyser and the coastal fog. It seems that *A. hypersalina* could be transported to and through various environments and is particularly adapted to the varying conditions, also within one salar, where changing salinities occur due to groundwater inflow. But rivers seem not to be important regarding placidid dispersal. *Haloplacidia cosmopolita* was isolated from the Salar de Atacama, where groundwater inflow also plays a particular role in salinity. *H. cosmopolita* was shown to be able to tolerate a wide range of salinities (4-200 PSU) and the genotype was detected in Salar de Huasco and Salar de Llamará but not in the groundwater or rivers. One possible explanation that placidids were not found in rivers could be that they cannot reach river systems due to the absence of outflows of the salars. We isolated two strains of *Wobblia pacifica* from different coastal rockpools (Chapter 1). In the Atacama region, the genotype of *Wobblia pacifica* was also detected within rockpools and the surrounded marine water, assuming species from the rockpools are closely related to other marine species. The genotype of the marine species *Wobblia lunata* could only be detected in marine coastal waters. This observation was also phylogenetically supported by representatives of the Atlantic Ocean, Japan and the Caribbean Sea, which formed a marine clade at the base of the phylogenetic tree, reflecting the marine origin of placidids. Placidids were frequently isolated from hypersaline environments also different species from one salar, which suggests allopatric as well as sympatric divergence processes with synchronous diversification. The combination of molecular features of protists together with dated geological events provided first insights into the divergence and therefore the evolution of heterotrophic flagellates from extreme environments. The evolution of protists correlated with the evolution of geological processes, especially within placidids. Even before the Atacama region developed, placidids (Stramenopiles) evolved from marine representatives (131 (77–196) Ma ago), and their separation from marine and hypersaline occurred around 114 (66-175) Ma ago. The uplift of the Western

Cordillera together with the resulting aridity and separation of the Atacama Desert about 20 Ma ago also correlates with the evolution of the athalassic linage *Allegra dunaii,* which could have evolved and diverged within this period. Placidids seem to be highly adapted to these extreme and isolated habitats and may be used as a model group to characterize these environments. Furthermore, the diversification could have taken place starting from the Salar de Atacama, the oldest isolated salar (Salisbury et al., 2011; Evenstar et al., 2016), with many divergence processes of placidids within the last 6 Ma. The different separated salars and their especially adapted heterotrophic protists were suitable for observing the evolution on a small scale. Due to the rare but existing exchange, it seems to be possible to observe diversification of the various organisms due to their high reproduction rate, ability to adapt to extreme conditions, and cysts' formation. Concerning placidids, there might have been several phases of dissemination and adaptation that we could also recognize in our study based on the similar community composition, phylogenetic tree and due to the investigations of the molecular clock.

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Diese Arbeit widme ich meiner
Nichte Pauline Meyer und meinem Neffen Jakob Meyer.
Dass ihr Mut und ihre Ausdauer sie jede Hürde im Leben überwinden lassen
und ihre Neugier an neuen Dingen niemals aufhört sie zu begeistern.

Sub-publications and Records of Achievement

Chapter 1

Rybarski, A.E., Nitsche, F., Park, J.S., Filz, P., Schmidt, P., Kondo, R., Simpson, A.G., Arndt, H., **2021**. Revision of the phylogeny of Placididea (Stramenopiles): Molecular and morphological diversity of novel placidid protists from extreme aquatic environments. European Journal of Protistology, https://doi.org/10.1016/j.ejop.2021.125809.

The study was designed and planned by AR together with Prof. Dr. Hartmut Arndt. The author performed sampling, and most parts of cultivation, isolation of cultures and rDNA sequencing. AR carried out the transcriptome analyses, light- and electron microscopical studies, salinity experiments, and phylogenetic analyses. Cultures and morphological data were also obtained by the co-authors Paulina Filz and Patricia Schmidt. Comparative cultures were also provided by the co-authors Jong Soo Park, Ryuji Kondo, and Alastair GB Simpson. The author mainly wrote the article under the guidance of Prof. Dr. Hartmut Arndt.

Chapter 2

Carduck, S., Nitsche, F., **Rybarski, A.,** Hohlfeld, M., Arndt, H., **2021**. Diversity and phylogeny of percolomonads based on newly discovered species from hypersaline and marine waters. European Journal of Protistology, 80, 125808.

The study was designed and planned by Sarah Carduck and Prof. Dr. Hartmut Arndt. AR was involved in sampling, cultivation, performing ecological experiments, and proofreading the manuscript.

Chapter 3

Schoenle, A., Hohlfeld, M., **Rybarski, A**., Sachs, M., Meyer, C., Schmidt, P., Freches, E., Wiechmann, K., Arndt H, **manuscript**. *Cafeteria* in extreme environments: description and ecological investigations on *C. burkhardae* and three new species (*C. baltica, C. biatacamiensis, C. paulosalfera*) from the Atacama Desert and the deep ocean.

The study was designed and planned by Alexandra Schoenle, Manon Hohlfeld, AR, and Prof. Dr. Hartmut Arndt. AR was involved in sampling, conducting morphological and ecological experiments, and proofreading the manuscript.

Chapter 4

Rybarski, A.E., Nitsche, F., Schoenle, A, Voigt C, Staubwasser M, Arndt H., **manuscript**. High diversity and isolated distribution of aquatic protists in salars of the Atacama Desert at extremely high salinities

The study was designed and planned by AR together with Prof. Dr. Hartmut Arndt. AR performed sampling, molecular investigations and bioinformatic evaluation. The bioinformatic approach was also supported by Alexandra Schoenle and Frank Nitsche. Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) was mainly conducted by Claudia Voigt and Michael Staubwasser; AR conducted the sampling for this approach and the preparation of samples. AR mainly wrote the manuscript under the guidance of Prof. Dr. Hartmut Arndt.

Chapter 5

Rybarski, A.E., Nitsche, F., Schoenle, A, Voigt C, Staubwasser M, Arndt H., **manuscript**. Biodiversity and distribution of protists via the water cycle in the Atacama - metabarcoding analyses of various natural water transport systems in northern Chile.

The study was designed and planned by AR together with Prof. Dr. Hartmut Arndt. AR performed sampling, molecular investigations, and most parts of bioinformatic evaluation. Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) was conducted by Claudia Voigt and Michael Staubwasser; AR conducted the sampling for this approach and the preparation of samples. AR mainly wrote the manuscript under the guidance of Prof. Dr. Hartmut Arndt.

Chapter 6

Arndt, H., Ritter, B., **Rybarski, A.**, Schiwitza, S., Dunai, T., Nitsche, F., **2020**. Mirroring the effect of geological evolution: Protist divergence in the Atacama Desert. Global and Planetary Change, 190, 103193.

The study was designed and planned by Prof. Dr. Hartmut Arndt and Dr. Frank Nitsche. AR was involved in the sampling, cultivation, isolation, provided transcriptomic data of all placidids, and proofread the manuscript.

Erklärung gemäß § 7 Absatz 8 der Promotionsordnung

„Hiermit versichere ich an Eides statt, dass ich die vorliegende Dissertation selbstständig und ohne die Benutzung anderer als der angegebenen Hilfsmittel und Literatur angefertigt habe. Alle Stellen, die wörtlich oder sinngemäß aus veröffentlichten und nicht veröffentlichten Werken dem Wortlaut oder dem Sinn nach entnommen wurden, sind als solche kenntlich gemacht. Ich versichere an Eides statt, dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie - abgesehen von unten angegebenen Teilpublikationen und eingebundenen Artikeln und Manuskripten - noch nicht veröffentlicht worden ist sowie, dass ich eine Veröffentlichung der Dissertation vor Abschluss der Promotion nicht ohne Genehmigung des Promotionsausschusses vornehmen werde. Die Bestimmungen dieser Ordnung sind mir bekannt. Darüber hinaus erkläre ich hiermit, dass ich die Ordnung zur Sicherung guter wissenschaftlicher Praxis und zum Umgang mit wissenschaftlichem Fehlverhalten der Universität zu Köln gelesen und sie bei der Durchführung der Dissertation zugrundeliegenden Arbeiten und der schriftlich verfassten Dissertation beachtet habe und verpflichte mich hiermit, die dort genannten Vorgaben bei allen wissenschaftlichen Tätigkeiten zu beachten und umzusetzen. Ich versichere, dass die eingereichte elektronische Fassung der eingereichten Druckfassung vollständig entspricht."

Datum: 03.01.2022

Unterschrift: 

Alexandra Eleonore Rybarski

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D-50354 Hürth

Germany

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Email: alexandra.rybarski@gmail.com

Biologist specialized in protistology

EDUCATION

Since 10-2017	**PhD Student**, University of Cologne, Germany. Thesis: "Biodiversity and Evolution of Protists in the Atacama Desert"
2014-2017	**Master's degree**, Master in Biology, University of Cologne, Germany Thesis: "Analysis of evolutionary processes within the placidid stramenopile flagellates "
2010-2014	**Bachelor's degree**, Bachelor in Biology, University of Cologne, Germany Thesis: "Preliminary studies on the diversity of protists in extreme hypersaline environments"

SKILLS AND QUALIFICATION

Language	German: Native speaker, English: fluently, Spanish: of reason knowledge, Latin (Latinum)
Laboratory	• Extensive experience in molecular biology (Sanger sequencing, Illumina HiSeq (RNA/DNA), cloning, Illumina NovaSeq, metabarcoding). • Knowledge in light microscopy, transmission electron microscopy, as well as high-resolution DIC microscopy.

Informatics	• Illumina NGS: FastQC, Trimmomatic, Trinity, Bowtie, Samtools • Overview of biological databases • Molecular biological characterisation programs (BioEdit, SeaView, Clustal Omega, PhyML, RaxML, Mr Bayes) • Knowledge of the operating systems Linux, MacOS, Windows • Additional knowledge: Word, Excel, PowerPoint (very good), R and RStudio (good).
Further skills	• Event management according to § 38 Abs. 2 MVStättVO or the corresponding state law • Completed examination of the German Chamber of Industry and Commerce concerning expert security knowledge according to § 34a GewO. • One-year education as a mediator in conflict situations.

CONTRIBUTIONS TO NATIONAL / INTERNATIONAL

2021-02	40th Annual Meeting of the German Society for Protozoology (DGP 2021), Essen, Germany. High diversity and the adaptation of aquatic protists to extreme conditions in the Atacama Desert. Rybarski AE, Nitsche F, Voigt C, Staubwasser M, Schoenle A, Arndt H.
2021-01	Ecological Colloquium of the Institute of Zoology, Cologne, Germany High diversity and the adaptation of aquatic protists to extreme conditions in the Atacama Desert. Rybarski AE, Nitsche F, Voigt C, Staubwasser M, Arndt H.
2020_02	39th Annual Meeting of the German Society for Protozoology, Kaiserslautern, Germany Biodiversity and adaptation of protists to extreme aquatic environments in the Atacama Desert. Rybarski AE, Nitsche F, Voigt C, Staubwasser M, Arndt H.
2019-07	VIII European Congress of Protistology – ISOP joint meeting, Rome, Italy Biodiversity and adaptation of placidid protists to extreme aquatic environments in the Atacama Desert. Rybarski AE, Nitsche F, Arndt H.

2019-06 Ecological Colloquium of the Institute of Zoology, Cologne, Germany
High diversity and the adaptation of aquatic protists to extreme conditions in the Atacama Desert. Rybarski AE, Nitsche F, Voigt C, Staubwasser M, Arndt H.

2019-02 38th Annual Meeting of the German Society for Protozoology, Vienna, Austria
Biodiversity and adaptation of protists to extreme aquatic environments in the Atacama Desert. Rybarski AE, Nitsche F, Filz P, Hohlfeld M, Arndt H.

2018-03 IV Workshop en Ecología Microbiana „Revolución microbiana y diversidad", Universidad de Antofagasta, Chile
Global diversity of placidid protists from saline inland waters. Rybarski AE, Carduck S, Nitsche F, Arndt H.

2018-02/03 37th Annual Meeting of the German Society for Protozoology, Cologne, Germany
Analysis of evolutionary processes within the placidid stramenopile flagellates. Rybarski AE, Nitsche F, Arndt H.

- Head of the organisation committee
- Session Chair: Protist Ecology II

2017-02 36th Annual Meeting of the German Society for Protozoology, Meißen, Germany
Comparison of the biodiversity of Placididea in regard to their geographical and genetical distribution. Rybarski AE, Carduck S, Nitsche F, Arndt H.

2016-09 32nd Annual Meeting of the German Society for Limnology e.V., Vienna, Austria
New heterotrophic flagellates of Chilean inland waters. Rybarski AE, Carduck S, Nitsche F, Arndt H.

2015-03 34th Annual Meeting of the German Society for Protozoology, Magdeburg, Germany
Enlarging the halotolerant group of Placididea (Stramenopila): A new heterotrophic flagellate from Chilean inland waters. Rybarski AE, Carduck S, Jeuck A, Nitsche F, Arndt H.

PUBLICATIONS

- **Rybarski, A.E.,** Nitsche, F., Voigt, C., Staubwasser, M., Arndt, H. (in preparation). Biodiversity and distribution of protists via the water cycle in the Atacama - metabarcoding analyses of various natural water transport systems in northern Chile
- Schoenle, A., Hohlfeld, M., **Rybarski, A**., Sachs, M., Freches, E., Wiechmann, K., Nitsche, F., Arndt H. (in preparation). *Cafeteria* in extreme environments: description and ecological investigations on *C. burkhardae* and three new species (*C. baltica, C. atacamiensis, C. paulosalfera*) from the Atacama Desert and the deep ocean
- **Rybarski, A.E.,** Nitsche, F., Schoenle, A., Voigt, C., Staubwasser, M., Arndt, H. (in preparation). High diversity and isolated distribution of aquatic protists in salars of the Atacama Desert at extremely high salinities
- **Rybarski, A.E.,** Nitsche, F., Park, J.S., Filz, P., Schmidt, P., Kondo, R., Simpson, A.G., Arndt, H. (2021). Revision of the phylogeny of Placididea (Stramenopiles): molecular and morphological diversity of novel placidid protists from extreme aquatic environments. European Journal of Protistology, p.125809.
- Carduck, S., Nitsche, F., **Rybarski, A.,** Hohlfeld, M., Arndt, H. (2021). Diversity and phylogeny of percolomonads based on newly discovered species from hypersaline and marine waters. European Journal of Protistology, 80, 125808.
- Arndt, H., Ritter, B., **Rybarski, A.**, Schiwitza, S., Dunai, T., Nitsche, F. (2020). Mirroring the effect of geological evolution: Protist divergence in the Atacama Desert. Global and Planetary Change, 190, 103193.
- **Rybarski, A.E.**, Arndt, H. (2018). Report of the 2018 meeting of the German Society for Protozoology

AWARDS

2020-03	**Best Poster Award:** 39th Annual Meeting of the German Society for Protozoology, Kaiserslautern, Germany. 2nd Place
2017-02	**Oral Presentation Award:** 36th Annual Meeting of the German Society for Protozoology, Meißen, Germany. 2nd Place

MEMBERSHIPS

2021	European Geosciences Union (EGU)
Since 2018	Founder and chair of the ecological and non-profit association "BlechWech"
Since 2017	German Society for Protozoology
Since 2017	International Society of Protistology

WORKSHOPS

2019	Digital Microscopy workshop
2017-02	Microscopy and Taxonomy workshop in Microalgae on behalf of the German Society for Protozoology
2007	Participation in the practical seminar for youth initiatives of the German Youth for Europe, Hannover

INTERNSHIPS AND STAYS ABROAD

2019-02	Expedition to Chile
2018-03/04	Expedition to Chile, Bolivia and Peru.
2015-09	Terrestrial excursion to southern France: Co-supervision and support in the determination of organisms.
2015-03/04	Expedition to Chile.
2014-08/09	Expedition to Tanzania. Working title: Insects of Tanzania.
2014-04	Pilgrimage to Israel and Palestine.
2013-05	Internship in marine biology on island Helgoland, Germany.

2013-03	Desert expedition to Namibia. Working title: Tenebrionidae of Namibia.
2012-08/09	External internship: Turtle Foundation, Volunteer, Cape Verde.

FURTHER EXPERIENCES

2018/2019	Assistant during the advanced module: Experimental Ecology.
2016	Assistant during the Master's module: Methods and Theoretical Concepts in Ecology.
2013-2014	Member of the appointment committee of the W2 Prof. for Zoology of the University of Cologne (Succession Schierenberg).
2013-2014	Member of the appointment committee of the W3 Prof. for Zoology of the University of Cologne (Succession Berking).
2014, 2016, 2017	Assistant during the basic studies modul of Biology IV (Ecology). Botanical and zoological part.
2012-2013	Tutor of the programme for first-year students of the student body of biologists at the University of Cologne.
2011-2012, 2013-2015	Co-Tutor of the programme for first-year students of the student body of biologists at the University of Cologne.
2010-2020	Acclaimed member of the student body of biologists at the University of Cologne. 2012-2020: Financial consultant.