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Molecular and Morphological Snapshot Characterisation of the Protist Communities in Contrasting Alpine Glacier Forefields

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Abstract. Phagotrophic protist diversity in oligotrophic soils such as alpine glacier forefields is still poorly studied. Combining morphologic observations with molecular-based analyses, we assessed the diversity of major phagotrophic protist groups in two contrasting glacier forefields in the Swiss Alps (Tiefen glacier forefield, siliceous bedrock, and Wildstrubel glacier forefield, calcareous bedrock), at sites differing in soil development. Ciliates and heterotrophic flagellates could be detected with both approaches, while amoebae could be observed only microscopically. Soils from Tiefen and Wildstrubel glacier forefields harboured distinctly different ciliate, flagellate and amoebae communities. The ciliate clone libraries from the Tiefen glacier forefield were dominated by Oligohymenophorea-related sequences while those from the Wildstrubel glacier forefield were dominated by Spirotrichea-related sequences. Testate amoebae morphospecies of the genera *Corythion, Cryptodifflugia, Euglypha* and *Tracheleuglypha* were restricted to the Tiefen glacier forefield, while *Centropyxis* and *Trinema* to the Wildstrubel one. No ciliate sequences and only a few ciliate and testate amoebae morphospecies could be retrieved from unvegetated soils of both glacier forefields. The ciliate and testate amoebae community detected at unvegetated sites were a subset of the community developed at vegetated sites. Overall, our results suggest that alpine glacier forefields are colonised by a diverse community of phagotrophic protists which seems to be shaped by bedrock geology and vegetation cover.

Key words: Ciliates, flagellates, testate amoebae, deglaciated soils, bedrock geology, siliceous soils, calcareous soils, vegetation cover.

INTRODUCTION

Areas exposed by receding glaciers are characterised by ecological succession processes, as the unvegetated exposed till and bedrock close to the glacier terminus is gradually colonised by plants. In a similar manner, microbial populations in glacier forefields undergo successional changes. Unvegetated alpine soils very close to the glacier front, which are characterised by oligotrophic conditions (Kaštovská *et al.* 2007, Schütte *et al.* 2009), harbour specialized heterotrophic bacterial and fungal communities that can cope with carbon and nutrient limitation (Nemergut *et al.* 2005, Lazzaro *et al.* 2009, Brankatschk *et al.* 2011, Zumsteg *et al.* 2012). Such microbial communities make a living on the allochthonous input of nutrients and organic matter (Hodkinson *et al.* 2002, 2003), as well as from an-

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cient organic carbon released from the receding glacier (Bardgett *et al.* 2007). Autotrophic bacteria successfully colonising unvegetated soils (i.e. nitrogen fixers, Brankatschk *et al.* 2011) tend to be replaced by decomposers of plant material (Schütte *et al.* 2009, Zumsteg *et al.* 2012), which become more important in vegetated soils.

Phagotrophic protists are considered to be initial colonisers of new soil surfaces such as Antarctic soils and fellfields (Smith 1996, Lawley *et al.* 2004) and recultivated soils after open cast mining activities (Wanner and Xylander 2005). Special adaptations such as extracellular shells and cyst formation allow phagotrophic protists to be distributed by wind, rain or animals and to outlast dry conditions although they strongly depend on water for survival (Foissner 1987). In soils, phagotrophic protists regulate microbial populations and influence key ecosystem processes (Coûteaux and Darbyshire 1998, Wetzel 2001, Bonkowski 2004). Nitrogen and phosphorus compounds released by phagotrophic protists promote the productivity of microbial populations (Griffiths 1994), and growth and reproductive success of plants (Bonkowski 2004, Krome *et al.* 2009). The diversity, abundance and succession of phagotrophic protists in deglaciated soils depend on different environmental factors, ranging from water content, porosity, organic matter (Anderson 2002, Foissner 1999), to the presence of plants (Smith and Tearle 1985, Frederiksen *et al.* 2001, Wanner and Xylander 2005). It is however still unclear if and how other site-related characteristics, such as bedrock geology, soil pH, nutrient quality and concentrations, may affect the presence of certain phagotrophic protist species and shape the protist community composition in deglaciated soils of glacier forefields. A recent study evidenced that bedrock geology mainly shapes the community composition of testate amoebae in unvegetated soils (Carlsson *et al.* 2010).

Traditionally, phagotrophic protists have been examined on the basis of their morphological and physiological features (Blackwelder 1967) and have been classified according to the morphospecies concept. However, microscopic identification is limited by recognizable morphological features and undersampling is a common risk (Foissner 2006). With the development of new molecular technologies, such as the profiling of the 18S rRNA gene or of the variable ITS region, it has been possible to characterise the community composition of different phagotrophic protist groups (Finlay 2004, Adl *et al.* 2012). Hence, we explored the diversity of major groups of phagotrophic protists, such as flagellates, amoebae and ciliates by combining the traditional microscopy approach and molecular profiling of the 18S rRNA gene.

Previous studies (Lazzaro *et al.* 2009, Brankatschk *et al.* 2011) demonstrated that bedrock geology modulates the structure of bacterial communities. In this work, we aimed to give a first snapshot characterisation and compare the phagotrophic protist communities (1) of two glacier forefields differing in bedrock geology, soil pH, nutrient quality and concentrations and (2) of recently deglaciated unvegetated soils and more developed, vegetated soils of glacier forefields.

MATERIALS AND METHODS

Study sites and sampling

Soil samples for the characterisation of soil properties and of the phagotrophic protist community composition were collected from 2 forefields of receding glaciers in the Swiss Alps, namely the siliceous forefield of Tiefen glacier (Canton Uri, Switzerland), and the calcareous forefield of Wildstrubel glacier (Canton Valais, Switzerland) (Fig. 1). A more detailed site description is given in Lazzaro *et al.* (2009) and Nauer *et al*. (2012). In July 2011, two transects were sampled at both glacier forefields differing in distance from the glacier front. Unvegetated transects corresponded to young, recently deglaciated, soils, of an approximate soil developmental age of < 5 yrs. Vegetated transects were located further away from the glacier front, where the first mosses and pioneer plants (i.e. *Leucanthemopsis alpina, Saxifraga* sp.) developed. Along each transect we selected 4 different sampling spots (replicates). Each replicate was obtained by pooling 5 subsamples collected within a range of 0.5 m at a depth of 2–5 cm. The soils were sieved on the spot through a 2-mm sieve. In total we obtained 16 samples (2 glacier types, 2 vegetation types, and 4 replicates). All the samples were stored in sterile plastic bags under cool conditions for transport. In the laboratory, the samples were partitioned into aliquots for physicochemical characterisation of the soils (stored at +4°C), for DNA-based analysis (stored at –20°C), and for microscopic observation (air dried and stored at room temperature in the dark).

Physicochemical characterisation of the soils

The soil samples were characterised in terms of their pH, water content, concentration and chemical composition of dissolved organic matter (DOC), soil organic matter (SOM), calcareous content, total carbon (TC), total nitrogen (TN) and soluble anions and cations.

Soil pH was measured in $CaCl_2$ (0.01 M) extracts with a Metrohm pH meter (Metrohm, Zofingen, Switzerland). Water content was estimated by weight loss after drying the samples overnight at 80°C.

For DOC analysis, 1 g soil was extracted with 10 ml of Milli-Q water, shaken on an overhead shaker for 1 h, and then filtered

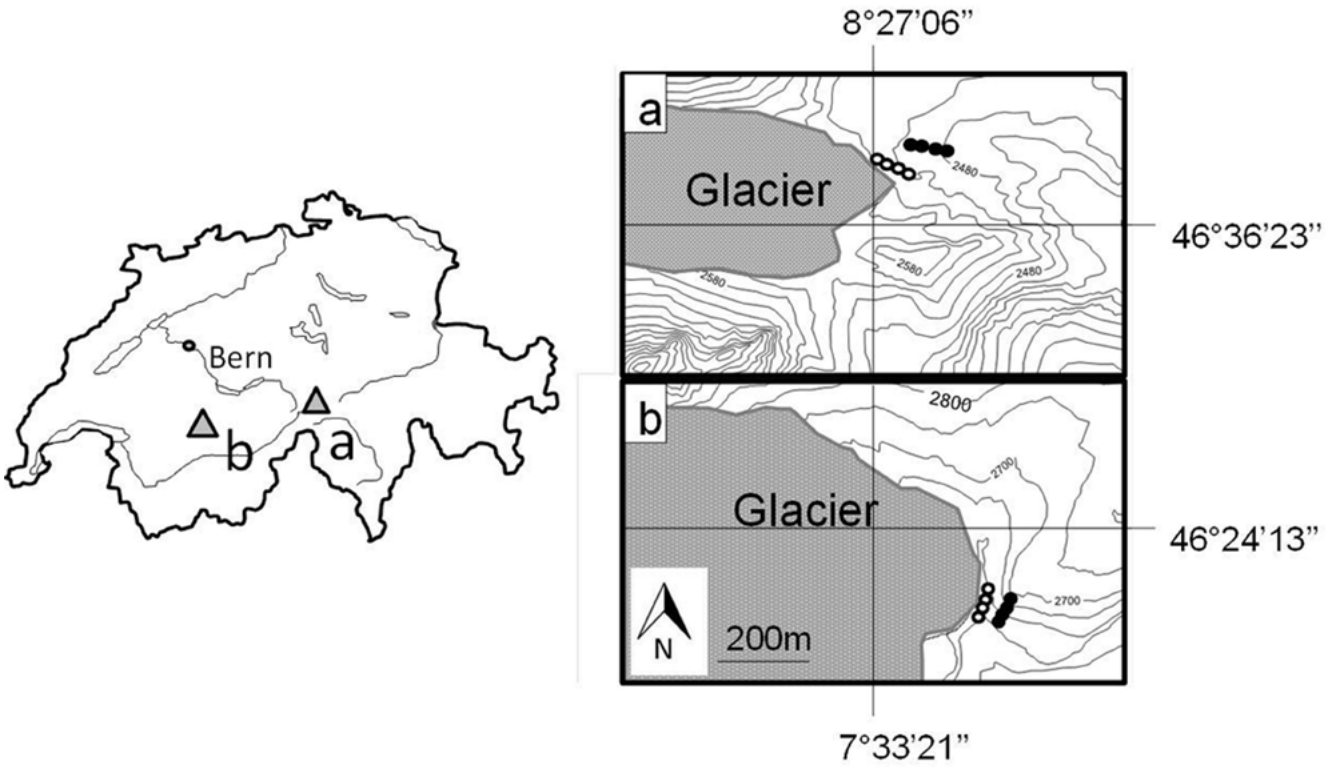

Fig. 1. Location of the two sampled forefields of the (a) Tiefen glacier and (b) Wildstrubel glacier. Dots indicate sampling spots (white: unvegetated transects; black: vegetated transects).

through a 0.45 µm nylon filter (Wicom Perfect Flow, Meienfeld, Switzerland). DOC was measured with a Shimadzu DOC analyser (Shimadzu, Reinach, Switzerland). Chemical composition of DOC in the water extracts was estimated with a Horiba Yvon Fluorolog®-3 (Horiba, Kyoto, Japan). To determine the SOM and calcareous content of the soil, a subsample of 20–40 g air dried soil was dried at 60°C to constant weight, ashed at 500°C and 900°C for 4 h each and weight after each step.

TC and TN were measured by incineration on an NC 1500 analyzer (CE Instruments, Wigam, UK), starting from 0.04–0.08 mg of dried and finely milled soil. For concentrations of soluble nutrients, 1 g of fresh soil was extracted with 5 ml of solutions of 0.01 M $CaCl_2$ (anions), 2 M KCl (cations) and 1 h shaking on an overhead shaker. After shaking, the samples were allowed to settle, and briefly centrifuged to remove soil particles. Anion (NO_3^-, SO_4^{2-}) concentrations were measured from the supernatant of the $CaCl_2$ extracts with a DX-320 ion chromatograph (Dionex, Sunnyvale, CA). Concentrations of NH_4^+ were measured colorimetrically on the supernatant of the KCl extracts according to Mulvaney *et al.* (1996).

Normality of data distribution was tested with the Shapiro-Wilk test. Significant differences of physicochemical parameters within and between sites were tested with the Kruskal-Wallis test of variance and the Mann-Whitney U-test. All statistical analyses were performed with Systat V. 12 software.

DNA extraction and PCR

DNA from the 16 soil samples was extracted with the MoBio Soil DNA extraction kit (Mobio, Carlsbad, CA) starting from 0.5 g of soil. DNA yields were estimated with Nanodrop (Thermo Fisher Scientific, Waltham, MA). For PCR with different phagotrophic protist targets (Table 1), 1−5 ng DNA were added to 25 µl of PCR reaction mix (including 1X DreamTaq Buffer, 0.5 µM of each primer, 0.2 mM dNTPs, 2.5 U DreamTaq in PCR-grade water). Primers (Table 1) were supplied by Microsynth (Balgach, Switzerland) and all other reagents by Fermentas (St. Leon-Rot, Germany). PCR conditions included a first step of denaturation at 95°C for 3 min, followed by 35 cycles of 95°C for 20 s, 30 s at the desired melting temperature (Table 1) and elongation at 72°C for 2 min. Reaction ended by a final elongation at 72°C for 4 min.

Clone libraries and sequencing analysis

For clone libraries of vegetated and unvegetated transects of Tiefen and Wildstrubel glacier forefields, PCR products from the 4 replicates within each transect were pooled and purified with a PCR purification kit (Qiagen, Hombrechtikon, Switzerland). Purified pooled PCR products were then quantified with Nanodrop before being ligated into the cloneJet vector (Fermentas) according to the manifacturers' instructions. Ligations were transformed by heat-

Table 1. Primers applied in the PCR. F: forward primer, R: reverse primer.

Target	Primer	Sequence 5'–3'	Melting temperature PCR (°C)	Reference
Ciliates	CS322F	GATGGTAGTGTATTGGAC	56	Puitika *et al.* 2007
	EU929R	TTGGCAAATGCTTTCGC		
Flagellates (*Heteromita globosa*)	Het F	TTGTCGGCCCACGGTTTCGT	55	Fredslund *et al.* 2001
	Het R	GAATCCTTTGTCGAACTATTTAGC		

shock into TOP10 competent cells (Promega AG, Dübendorf, Switzerland). Positive transformants, corresponding to cells able to grow on LB-ampicillin plates, were picked and transferred to 96-well plates containing LB-ampicillin medium (for storage) and 96-well plates containing 25 µl of PCR mix as described before, but containing 100 µM of vector-specific primers pJetF and pJetR (Fermentas).

PCR products of each clone were screened through RFLP analysis. PCR products were divided in two aliquots of 10 µl, which were digested with the restriction enzymes HaeIII (Fermentas) or AluI (Promega) for 3 h at 37°C. Digestion Master mix contained 0.2 µl of the desired enzyme, 2 µl of enzyme-specific buffer (Y+ buffer for HaeIII and R Buffer for AluI) and 7.8 µl of PCR-grade water. Digestions were visualised on 2.5% agarose gels by running 1.5 h at 60 V. Screening of the RFLP patterns was performed with the gel visualization software Genmarker ® (Softgenetics, State College, PA).

Selected clones were PCR-amplified again with the vector-specific primers and purified with Sephadex G-50 columns (Sigma-Aldrich, Buchs, Switzerland) as described in Lazzaro *et al*. (2011). PCR products were checked for concentration and purity with Nanodrop. Cycle sequencing was performed with 3 µl of purified PCR (corresponding to approximately 150 ng DNA) in 3 µl of a sequencing master mix containing 1 µl of 5X sequencing buffer, 1 µl of 1 µM vector-specific primer and 1 µl of Big Dye Terminator V1.1 (Applied Biosystems, Carlsbad, CA). Cycle sequencing reactions applied a first step of 94°C for 2 min, followed by 60 cycles at 94°C for 20 sec, 50°C for 5 sec and 60°C for 3 min. After removal of unincorporated dye through Sephadex columns, sequences were obtained with an ABI 3130XL capillary electrophoresis system using POP7 as a running polymer.

Sequences corresponding to each clone library were aligned with Bioedit V.7.0.9.0 (Hall 1999) and checked for chimeras with the Pintail chimera detection software V.1 (Ashelford *et al*. 2005). Rarefaction analysis with Chao1 non-parametric richness estimator was performed with the EstimateS software (Colwell 1997). Sequences were submitted to the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov) nucleotide database (accession numbers KC513787–KC513818 for ciliates and KF690710–KF690727 for flagellates).

Microscopic observation

We applied two approaches to microscopically observe phagotrophic protists of glacier forefields. The 4 replicate samples of each transect were pooled before preparing the enrichment cultures. In a first approach, 3–5 g of air dried soil of a pooled sample was placed in a sterile glass dish (sterilization at 180°C for 6 h) and wetted with approximately 15–25 ml of Volvic mineral water. Additional enrichment cultures were prepared after the 'non-flooded Petri dish method' (see Foissner 1987) to avoid the artificial pond milieu of the former method (Pussard 1967), where 10–50 g air dried soil were placed in a sterile glass dish and saturated but not flooded with deionized water (obtained by reverse osmosis). Directly after rewetting the soils, the culture liquid was carefully mixed and a small volume was placed on a glass slide covered with a cover slip and scanned for floating empty tests of testate amoeba that typically contain gas bubbles (Schönborn 1986).

Thereafter, enrichment cultures were incubated at 18°C and observed at least once a week for a total of 5 weeks. At each observation, soil and culture liquid were carefully mixed. The culture was left for some minutes to let large soil particles settle and then a subsample of the culture liquid was placed in an Utermoehl chamber or on a glass slide and scanned for phagotrophic protists. Additionally, a cover slip (sterilized by 90% ethanol and rinsed twice in autoclaved deionized water) was exposed on the surface of each culture liquid and scanned after > 3 h. At each observation, 3–4 subsamples were scanned at an upright (Axioplan, Zeiss, Jena, Germany) and inverse (Ti-U Eclipse, Nikon, Düsseldorf, Germany) microscope equipped with differential interference and phase contrast optics. Observed cells were video recorded or pictures were taken with a CCD camera for verification of species determination. Ciliate morphospecies were identified using the keys of Kahl (1930–1935), Foissner (1987, 1993) and Foissner and Berger (1996), testate amoeba morphospecies using Bonnet and Thomas (1960), and flagellate morphospecies using Patterson (2003).

RESULTS

Physicochemical characteristics

The physico-chemical characteristics of the soil samples reflected the different geological nature of the underlying bedrock at the two glacier forefields (Table 2). The pH was slightly alkaline 7.4 ± 0.1 (mean ± SD) in the soil samples from Wildstrubel glacier forefield, while a slightly acidic pH of 5.3 ± 0.3 was observed in the soil samples from Tiefen glacier forefield. Within the Tiefen glacier forefield, pH was slightly lower in the vegetated transect compared to the unvegetated transect (Table 2).

DOC concentration was 1–2 orders of magnitude higher ($p < 0.01$) in soils of the Wildstrubel than those of the Tiefen glacier forefield (Table 2). DOC concentrations were similar at the two transects at Wildstrubel glacier forefield, while the vegetated transect at Tiefen glacier forefield showed 10-times higher DOC concentrations ($p < 0.01$) than the unvegetated transect. The chemical composition of DOC determined by Fluorolog analysis (Table 2) differed between the two studied glacier forefields. The three DOC fractions (proteinaceous material, fulvic acids and humic acids) were similar in unvegetated and vegetated siliceous soils of the Tiefen glacier forefield. In all transects, except the unvegetated transect from Wildstrubel glacier forefield, fulvic acids made up more than 60% of the DOC pool. In the unvegetated transect from Wildstrubel glacier forefield, DOC showed a significantly higher relative abundance of the proteinaceous-related fraction than in the vegetated transect. SOM content was 2.5–3.0 times higher and TC content was 2 orders of magnitude higher in the calcareous soils from Wildstrubel glacier forefield than in the siliceous soils from Tiefen glacier forefield (Table 2). TN concentration was low in all samples. In the soil from Wildstrubel glacier forefield, concentration of NO_3^--N was significantly higher in the vegetated than in the unvegetated transect, while concentration of SO_4^{2-}-S tended to be lower and highly variable in the vegetated transect. In the siliceous soils from Tiefen glacier forefield, NH_4^+-N and SO_4^{2-}-S concentrations were similar in both transects.

Table 2. Soil characteristics of the studied transects (mean ± 1SD, n = 4) and analysis of variance (Kruskal-Wallis test of variance and Mann-Whitney U-test). UVT: unvegetated transect, VT: vegetated transect, < bdl: below detection limit (1 µM). Asterisks indicate significant differences at $p < 0.05$ (*) or < 0.001 (**). n.s.: not significant; n.a.: not available.

	Tiefen (T)		Wildstrubel (W)		Within site comparison		Between site comparison	Between vegetation comparison
	UVT	VT	UVT	VT	T	W		
pH (soil in $CaCl_2$)	5.6 ± 0.3	5.3 ± 0.3	7.6 ± 0.1	7.4 ± 0.1	n.s.	n.s.	**	n.s.
TC (%)[a]	0.06	0.06	8.4	8.0	n.a.	n.a.	n.a.	n.a.
SOM (mg · g soil dry weight^{-1})	2.0 ± 0.2	2.2 ± 1.2	5.5 ± 0.1	5.1 ± 0.0	n.s.	**	**	n.s
$CaCO_3$ (mg · g soil dry weight^{-1})	3.3 ± 0.2	3.2 ± 0.2	30.1 ± 0.1	30.2 ± 0.0	n.s.	n.s.	**	n.s
TN (%)[a]	0.02	0.02	0.02	0.02	n.a.	n.a.	n.a.	n.a.
NH_4^+-N (µg · g soil dry weight^{-1})	0.98 ± 0.18	1.13 ± 0.33	0.70 ± 0.34	0.74 ± 0.07	n.s.	n.s.	n.s.	n.s
NO_3-N (µg · g soil dry weight^{-1})	< bdl	< bdl	0.26 ± 0.23	0.77 ± 0.30	n.a.	n.a	n.a.	n.a.
SO_4^2-S (µg · g soil dry weight^{-1})	0.15 ± 0.02	0.13 ± 0.02	0.66 ± 0.27	0.45 ± 0.10	n.s.	n.s.	**	n.s.
DOC (µg · g soil dry weight^{-1})	0.05 ± 0.03	0.40 ± 0.30	2.98 ± 1.60	4.38 ± 2.29	**	n.s.	**	**
Proteinaceous DOC (%)	10.8 ± 2.7	11.5 ± 3.2	26.3 ± 26.9	6.2 ± 1.0	n.s.	**	*	n.s
Fulvic acids (%)	60.9 ± 4.8	63.5 ± 3.2	53.9 ± 22.5	65.7 ± 25.0	n.s.	n.s.	*	n.s
Humic acids (%)	28.3 ± 2.0	25.0 ± 1.3	19.8 ± 9.3	27.7 ± 13.0	n.s.	**	n.s.	n.s

[a] Data derived from one measurement of a pooled sample per transect.

Diversity of ciliates determined by molecular profiling

Positive PCR amplification with ciliate-specific primers was obtained for the samples of the vegetated transects but not for those from the unvegetated transects. For the clone library of each vegetated transect, 192 clones were screened by RFLP. Sequencing of randomly selected clones with distinct restriction patterns retrieved 19 phylotypes for the Tiefen glacier forefield and 18 for the Wildstrubel glacier forefield (see supplementary information available on the Journal's homepage). Ciliate-related clone libraries of the 18S rRNA gene evidenced a high diversity of ciliate species in vegetated transects from Tiefen and Wildstrubel glacier forefields (Fig. 2). The populations detected in the soils from Tiefen glacier forefield were distinct from those detected in Wildstrubel glacier forefield. Rarefaction analysis indicated that the number of sequenced clones covered a higher estimated diversity in the soil of Wildstrubel than Tiefen glacier forefield (Fig. 3). However, neither of the curves reached saturation. The Chao I richness estimator calculated a total richness of 31 phylotypes for the Wildstrubel glacier forefield sample, and 61 phylotypes for the Tiefen glacier forefield sample. Clone libraries covered 29.5% and 61.2%

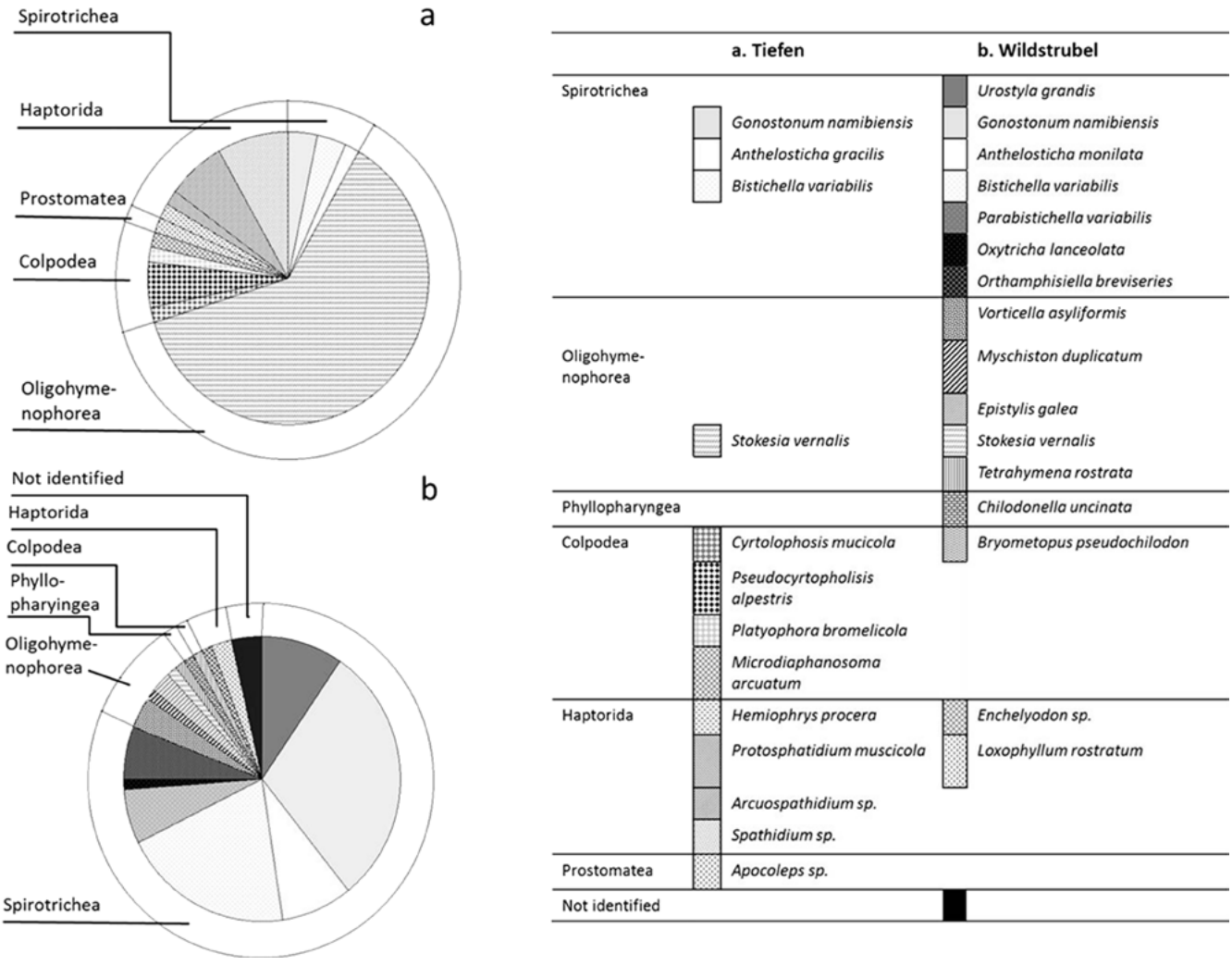

Fig. 2. Relative abundances (in percentage) of ciliate-related sequences detected in the 18S rRNA gene clone libraries from the vegetated transects of the (a) Tiefen forefield and (b) Wildstrubel forefield. Species names are based on BLAST comparison of the sequences with the NCBI database (first similarity with a known taxonomic group).

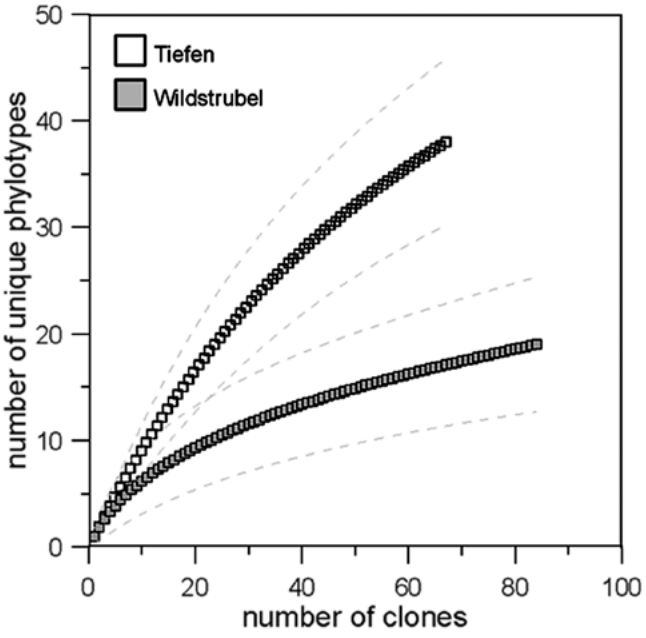

Fig. 3. Rarefaction analysis derived from the clone libraries of the vegetated transects of Tiefen forefield and Wildstrubel forefield. Dashed lines correspond to 95% confidence intervals.

of the estimated richness in samples from Tiefen and Wildstrubel glacier forefields, respectively.

In the clone library retrieved from Tiefen glacier forefield, Oligohymenophorea were by far the most represented group detected, with a relative abundance of 61% of all the sequences obtained (Fig. 3). All sequences of Oligohymenophorea showed a 90–92% similarity to *Stokesia vernalis* (Peniculia, accession number HM030738). The next most abundant group, Haptorida (Spathidiidae), made up 17% of the ciliate community at this transect and were mainly represented by sequences highly similar to *Arcuospathidium* (accession number JF263443). Colpodea-related (family Cyrtolophosididae) sequences had a relative abundance of 8.5%, while the remaining affiliations had a relative abundance < 3.5%.

The most dominant group detected in the clone library from Wildstrubel was highly similar to Spirotrichea. Urostyloidea, Oxytrichidae, and Trachelostylida made up to 80.9% within the Spirotrichea. Most sequences were closely related to *Gonostomum namibiensis* and to *Urostyla grandis* (accession numbers AY498655 and EF535731). Stichotrichia appeared to have a relative abundance of 27.4%, which was represented mainly by sequences related to *Bistichella variabilis* (accession number HQ699895). The next most abundant group belonged to Oligohymenophorea (mainly Peritrichia), and made up to 6% of the ciliate community, with sequences closely related to *Myschiston duplicatum* and *Epistylis galea* (accession numbers JN836352 and AF401527).

Diversity of flagellates and amoebae determined by molecular profiling

PCR amplification with *Heteromita globosa*-specific primers (Table 1) was successful in most of the samples (Table 3), except in the unvegetated transect from Tiefen glacier forefield. Sequencing of the related PCR products (see Supplementary Table S1, available online on the Journal's homepage), detected a total of 18 distinct phylotypes related to the novel order of Glissomonadida (gliding flagellates). The most common phylotypes sequenced appeared similar (in a range between 90% and 96% sequence similarity, Table S1) to uncultured eukaryotes found in cold habitats such as arctic and antarctic mats and antarctic moss pillars (i.e. accession numbers EU709212, EU7090189, and JN207853).

PCR-based analysis of amoebae appeared impaired by unspecific amplification of the 18S rRNA gene.

Diversity of ciliate morphospecies determined by microscopy

By microscopic observation after enrichment cultivation we detected a total of 25 ciliate morphospecies (Table 4). Twenty-two ciliate morphospecies were observed in enrichment cultures from soils of Tiefen glacier forefield and only 15 in soils of Wildstrubel glacier forefield. Both glacier forefields had 12 morphospecies in common, while 10 and 3 morphospecies were exclusively detected in soil enrichment cultures from either Tiefen or Wildstrubel glacier forefields, respectively. In contrast to the molecular profiling, ciliate morphospecies were detected in both unvegetated and vegetated transects (Table 3). More ciliate morphospecies were detected in enrichment cultures from vegetated than unvegetated soils of both glacier forefields (Table 4). Overall, soil enrichment cultures from all studied transects had two ciliate morphospecies in common. Most of the ciliate morphospecies detected in enrichment cultures of unvegetated soils were also recorded in vegetated soils. The vegetated transects of the two studied glacier forefields had a total of 12 ciliate morphospecies in common.

Table 3. Comparison between results of PCR amplification with different primer sets and morphospecies detected by microscopic observation. The sign – indicates negative PCR products or no microscopic observations. UVT: unvegetated transect, VT: vegetated transect. The sign + indicates positive PCR products or microscopic observations. na: not applicable.

	PCR-based analyses				Microscopic observation			
	Tiefen		Wildstrubel		Tiefen		Wildstrubel	
	UVT	VT	UVT	VT	UVT	VT	UVT	VT
Ciliates	–	+	–	+	+	+	+	+
Flagellates (*Heteromita*)	–	+	+	+	+	+	+	+
Amoeba (Testate)	na	na	na	na	+	+	–	+

Table 4. Ciliate morphospecies in soil enrichment cultures from Tiefen and Wildstrubel glacier forefields. Classification based on Adl *et al.* (2005, 2012). UVT: unvegetated transect, VT: vegetated transect. The sign + indicates presence and – indicates absence of morphospecies.

	Tiefen		Wildstrubel	
	UVT	VT	UVT	VT
Colpodea				
Colpoda cucullulus	+	–	–	–
Colpoda ecaudata	–	+	–	+
Colpoda inflata	–	–	–	+
Colpoda maupasi	+	+	–	+
Colpoda steinii	+	+	+	+
Cyrtolophosis elongata cf	–	+	+	+
Cyrtolophosis muscicola	+	+	+	+
Oligohyemophorea				
Cinetochilum margaritaceum	–	–	+	+
Cyclidium cf *muscicola*	–	+	–	–
Cyclidium glaucoma	–	+	–	+
Cyclidium versatile	+	+	–	+
Homalogastra setosa	–	+	+	+
Nassophorea				
Leptopharynx costatus	–	+	–	+
Spirotrichea				
Gonostomum sp.	–	+	–	+
Holosticha/Anteholosticha	–	+	–	–
Stichotricha cf *aculeata*	+	+	–	–
Unidentified sp.1	–	+	–	–
Unidentified sp.2	–	+	–	+
Unidentified sp.3	–	+	–	–
Unidentified sp.4	–	+	–	+
Haptorida				
Spathiidae	+	+	–	–
Unidentified				
species 1	–	+	–	–
species 2	–	–	–	+
species 3	–	+	–	–
species 4	–	+	–	–
Total number of morphospecies	7	21	5	15

The ciliate morphospecies in enrichment cultures of both glacier forefields were mainly represented by Colpodea (28%), Spirotrichea (28%), and Oligohymenophorea (20%). Nassophorea (4%) were represented by the morphospecies *Leptopharynx costatus*, which colonised enrichment cultures from the vegetated transect of both glacier forefields (Table 4). Litostomatea (Spathiidae, 4%) were exclusively observed in siliceous soils of Tiefen glacier forefield. The Colpodea were represented by 5 morphospecies of the genus *Colpoda* and 2 morphospecies of the genus *Cyrtolophosis*. Most of the morphospecies of the Spirotrichea were detected in soil enrichment cultures from the siliceous Tiefen glacier forefield. Similar to molecular profiling, the genera *Gonostomum* and *Holosticha/Anteholosticha* were detected in vegetated soils. All detected Oligohymenophorea were small Scuticociliatida represented by 3, 1 and 1 morphospecies of the genera *Cyclidium*, *Cinetochilum* and *Homalogastra*, respectively.

Diversity of flagellates and amoebae morphospecies determined by microscopy

Flagellates and testate amoebae were observed in soils and soil enrichment cultures of both glacier forefields (Table 3). Naked amoebae were also observed in soil enrichment cultures, except in cultures from the unvegetated transect of the Wildstrubel glacier forefield. However, their diversity remained most probably underestimated by the applied approach. Flagellates such as *Heteromita*, *Bodo* and *Bodomorpha* were observed in all cultures. Most of the flagellates could however not be distinguished to the genus or species level. In addition, *Goniomonas* (Cryptophyceae, Chromalveolata), *Bicosoeca* and *Spumella* (Stramenopiles, Chromalveolata), *Monosiga* (Choanomonada, Opisthokonta), and morphospecies of Eubodonida and Neobodonida (Euglenozoa, Excavata) were detected in soil enrichment cultures from Tiefen and Wildstrubel glacier forefields. *Heteronema* and *Anisonema* (Euglenozoa, Excavata) as well

as *Heteromita*, *Bodomorpha* and *Cercomonas* (Cercozoa, Rhizaria) were exclusively found in soil enrichment cultures from the siliceous Tiefen glacier forefield.

The testate amoebae were represented by 8 morphospecies of the Amoebozoa, 12 morphospecies of the Rhizaria and 4 unidentified species (Table 5). The two glacier forefields of differing bedrock material had 2 morphospecies in common. No testate amoebae were detected in unvegetated soils of the Wildstrubel glacier forefield (Tables 3 and 5). Similarly, soil of the vegetated transect of the Tiefen glacier forefield inhabited more morphospecies (21) than the unvegetated transect (7).

The testate amoebae in soils and soil enrichment cultures of both glacier forefields were mainly represented by the Arcellinida (29.2%), Euglyphidae (29.2%) and Trinematidae (20.8%). Within the three groups, *Cryptodifflugia, Euglypha, Tracheleuglypha*, *Corythion* spp. and cf *Schwabia* sp. (Difflugidae) were exclusively observed in siliceous soils of the Tiefen glacier forefield (Table 5). Morphospecies of the genera *Centropyxis*, *Trinema* and one unidentified morphospecies were restricted to vegetated soils of the Wildstrubel glacier forefield.

DISCUSSION

Methodological considerations

By using a combination of microscopic observation and molecular approach we aimed at providing a first insight into the phagotrophic protist community in contrasting, recently exposed alpine glacier forefield soils. In the present study, we observed higher species richness in the 18S rRNA ciliate clone libraries than by microscopy, which is in agreement with previous studies (Massana *et al.* 2004, Dopheide *et al.* 2009). As many ciliate species are cryptic, molecular techniques usually reveal a greater diversity than morphological techniques (Finlay *et al.* 2006, Schmidt *et al.* 2007, Foissner *et al.* 2008), even in comparison to more sophisticated methods as silver staining and electron microscopy (Foissner *et al.* 2008). Furthermore, molecular profiling detects also species that are rare, adhere to soil particles or are represented in their encysted stage (hardly detectable by microscopy if they do not excyst in enrichment cultures).

While molecular-based approaches may overcome such cultivation biases (Moreira and López-Garcia 2002), it is still extremely difficult to obtain a complete picture of the overall diversity of phagotrophic protist communities in soils. In particular, the major challenge seems to be related in the molecular detection of amoebae diversity. While species-specific primers have been applied successfully to environmental samples (Bråte *et al.* 2010), good primer sets targeting the complete set of species of the phagotrophic protist community still needs to be developed (Pawlowski and Burki 2009, Smirnov *et al.* 2011). Promising results for marine environments have been shown from the high-throughput sequencing of the V4 and V9 SSU rRNA regions, obtained with primers targeting major eukaryotic groups (Stoeck *et al.* 2010). However, there are still open questions related to heterogeneous environments such as soils. In conclusion, the application of both methods, morphological observation and molecular detection, is necessary and yielded a more complete picture of the phagotrophic protist community in soils.

Phagotrophic protist community modulated by bedrock geology

Both, microscopic observations and clone libraries depicted the same diversity trends. A more diverse ciliate community occurred in siliceous soils of the Tiefen glacier forefield than in calcareous soils of the Wildstrubel glacier forefield (Fig. 2, Table 4). Similarly, more morphospecies of testate amoebae were detected by microscopic observations in siliceous soils of the Tiefen glacier forefield than in calcareous soils of the Wildstrubel glacier forefield. Previous studies demonstrated the differences occurring in glacier forefield bacterial communities inhabiting soil developed on calcareous or siliceous bedrock (Lazzaro *et al.* 2009, 2011). Soils from recently deglaciated soils are generally oligotrophic, but the geologic nature of the parent material is related to different soil pH, different soil texture and microbial niches. As seen with bacteria, bedrock geology of the studied glacier forefields seemed to affect the occurrence of certain phagotrophic protist species and determine community composition. RDA analysis showed that protist community composition of Tiefen and Wildstrubel glacier forefields were separated on the first axis, which was related to site-specific factors (Supplementary Fig. S1, available on the Journal's homepage). However, as the dataset used is a snapshot of the summer season, the role of bedrock geology in other alpine soils needs to be further verified.

Species numbers of ciliates and testate amoebae were in the same range as detected in soils of alpine habitats (10–50 ciliate morphospecies per site; Bamforth 1995) and a glacier forefield in Alaska (4–21 testate amoe-

Table 5. Testate amoeba morphospecies in soils and soil enrichment cultures from Tiefen and Wildstrubel glacier forefields. Classification based on Adl *et al.* (2005, 2012). UVT: unvegetated transect, VT: vegetated transect. The sign + indicates presence and – indicates absence of this morphospecies.

	Tiefen			Wildstrubel	
	UVT	VT		UVT	VT
Testate amoeba					
Amoebozoa, Tubulinea					
Centropyxis aculeata (soil type)	–	+		–	–
Centropyxis aerophila-complex	+	+		–	+
Centropyxis elongata	–	+		–	–
Centropyxis sphagnicola	+	+		–	–
Centropyxis cf *vandeli*	–	+		–	–
Cryptodifflugia minuta	–	+		–	–
Cryptodifflugia sp.	–	+		–	–
cf *Schwabia* sp.	–	+		–	–
Rhizaria (SAR), Cercozoa					
Euglypha anodonta	+	+		–	–
Euglypha compressa	–	+		–	–
Euglypha dolioliphormis	–	–		–	–
Euglypha laevis	–	+		–	–
Euglypha umbilicata	+	+		–	–
Tracheleuglypha acola	–	+		–	–
Trinema enchelys	–	–		–	+
Trinema lineare	+	+		–	+
Corythion dubium	+	+		–	–
cf *Corythion pulchellum*	–	+		–	–
Corythion cf *delamarei*	–	+		–	–
Pseudodifflugia gracilis var. *terricola*	+	–		–	–
Unidentified					
species 1	–	+		–	–
species 2	–	+		–	–
species 3	–	–		–	+
species 4	–	+		–	–
Total testate amoeba	7		21	0	4

bae morphospecies per site; Carlsson *et al.* 2010). At a broader taxonomic level, Colpodea, Spirotrichea and Oligohymenophorea were present in soils of both glacier forefields. Colpodea and Spirotrichea are dominant cultivable ciliate groups of Central Alp soils (Lüftenegger *et al*. 1985, Foissner 1987), other high altitude soils (Li *et al.* 2010), and forest soils (Foissner *et al.* 2005). Within the Oligohymenophorea, Peniculia and Peritrichia were restricted in the clone libraries from soils of the Tiefen and Wildstrubel glacier forefields, respectively. Neither group could be observed with microscopy. Sequences clustering with Peniculia were closely related (97–98%) to an uncultured, cold-adapted ciliate isolated from snow (accession number AB725340). The free-living Peritrichia are generally characterised by high species diversity (Foissner *et al.* 2010), and have been found in both, aquatic and soil environments.

Phagotrophic protist community in relation to vegetation cover

The physicochemical analysis of Tiefen and Wildstrubel glacier forefield soils showed differences in DOC and nutrient concentrations between unvegetated and vegetated transects, which indicate different nutrient quality and availability for the local microbial and phagotrophic protist communities. Few studies attempted to investigate diversity of phagotrophic protists in undeveloped and unvegetated soils (Frederiksen *et al.* 2001, Wanner and Elmer 2009). Phagotrophic protists colonise soils very rapidly, and complexity of phagotrophic protist communities increases enormously in a range of 10 years soil development (Smith 1996). The primary colonisers of glacier forefields and extreme cold environments at high altitudes are usually heterotrophic flagellates, naked and testate amoebae (Smith 1996, Bamforth *et al.* 2005). However, soil colonisation by testate amoebae appeared strongly related to bedrock geology, i.e. texture and moisture, and the presence of vegetation patches (Wanner and Elmer 2009, Carlsson *et al.* 2010). Unvegetated spots of a volcanic island in Iceland were colonised exclusively by heterotrophic flagellates (Frederiksen *et al.* 2001). Flagellates such as *Heteromita* sp. and *Bodo* spp. were abundant in both unvegetated and vegetated sites of the glacier forefields studied. *Heteromita globosa* resists freeze-thaw cycles through rapid encysting and excysting cycles (Smith 1996). Similarly, *Bodo* (i.e. *Bodo saltans*) is ubiquitously distributed (Foissner 1987).

Our data point out that ciliates and testate amoeba seemed to be dependent also on the presence of plants. In agreement with previous studies, cultivable ciliates are rare or absent from polar desert soils (Bamforth *et al.* 2005) and complexity of phagotrophic protist communities increases with soil development (Wanner and Xylander 2005). Phagotrophic protists are more abundant and diverse in the rhizosphere of plants where they accelerate root growth and uptake of mineralized nutrients (Bonkowski 2004, Krome *et al.* 2009). Our

microscopic determination revealed no clear indication of species replacement as also shown by Wanner and Xylander (2005). It seems evident that the ciliate and testate amoebae community at unvegetated sites was a subset of the community developed at vegetated sites of both glacier forefields.

In conclusion, both studied glacier forefields appeared to be colonised by distinctly different communities of phagotrophic protists. The specific bedrock geology, chemical characteristics of the local soil habitat and vegetation cover seemed to shape the phagotrophic protist community already at a high taxonomic level. Extremely oligotrophic environments such as calcareous soils and unvegetated sites of glacier forefields could potentially represent a selective pressure on the phagotrophic protist communities and filters for species with specific adaptations to this habitat.

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