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# Primer pairs for the specific environmental detection and T-RFLP analysis of the ubiquitous flagellate taxa Chrysophyceae and Kinetoplastea

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ABSTRACT

Bacterivorous protists play a key role in microbial soil food webs, however due to the lack of specific PCR protocols targeting selected protist taxa, knowledge on the diversity and dynamics of these groups is scarce. We developed specific PCR primers in combination with a T-RFLP protocol for the cultivation-independent analysis of two important taxa of bacterivorous flagellates, the *Chrysophyceae* and *Kinetoplastea*, in soil samples. Sequence analysis of clone libraries originating from two soils in temperate regions demonstrated the specificity of the respective primer pairs. Clone sequences affiliating to the *Chrysophyceae* mainly clustered within the clade C2, which has been known so far for its presence mainly in cold climatic regions, whereas *Kinetoplastea* sequences were mainly related to the *Neobodonid* clade. Based on an *in silico* restriction analysis of database sequence entries, suitable restriction enzymes for a T-RFLP approach were selected. This *in silico* approach revealed the necessity to use a combination of two restriction enzymes for T-RFLP analysis of the *Chrysophyceae*. Soil T-RFLP profiles reflected all T-RFs of the clone library sequences obtained from the same soils and allowed to distinguish flagellate communities from different sites. We propose to use these primer pairs for PCR detection and rapid fingerprint screening in environmental samples and envisage their use also for quantitative PCR or next generation sequencing approaches.



## 1. Introduction

There is a constantly rising interest in culture-independent methods allowing for the specific detection and analysis of protistan communities in the environment (Moreira and López-García, 2002; Wu et al., 2009; Bates et al., 2012). Most attention has been dedicated so far to approaches based on PCR-amplification and sequencing of 18S rRNA genes from environmental samples, thereby circumventing the known limitations of direct microscopic observation and cultivation of protists (Caron et al., 2004). In particular heterotrophic flagellates or amoebae are hardly recognisable in soil samples, while species identification often requires living material and electron microscopy. Furthermore "sibling species", i.e. genetically distant species displaying similar morphology, are almost impossible to identify (Boenigk, 2008). As a result of the increasing application of 18S rRNA gene based approaches new protistan phylotypes are constantly being revealed (Lopez-Garcia et al., 2001; Mangot et al., 2010; Taib et al., 2013) which is challenging our view on the diversity, distribution and functioning of eukaryotic microorganisms.

Most PCR-based environmental surveys conducted so far have applied general eukaryotic primer sets to cover a broad diversity of eukaryotic microorganisms. The majority of these surveys analysed samples from aquatic systems (e.g. Lopez-Garcia et al., 2001; Triadó-Margarit and Casamayor, 2012), while only a few studies extended the application of rRNA-based methods to protistan communities in soil (Lawley et al., 2004; Fell et al., 2006; Moon-van der Staay et al., 2006; Bates et al., 2012). However, it is reasonable to assume that the use of group-specific primers might reveal more diversity and allow for a more comprehensive representation of protistan groups in environmental surveys (Bass and Cavalier-Smith, 2004; Lara et al., 2007; Jousset et al., 2009; Lejzerowicz et al., 2010).

Heterotrophic flagellates represent an important group of bacterivorous protists, i.e. protists feeding on bacteria. They are crucial parts of nutrient cycles transferring bacterial biomass to higher trophic levels (Ekelund and Rønn, 1994; Bonkowski, 2004; Adl et al., 2006). In particular flagellates smaller than 20 μm show very high metabolic activity (Fenchel, 1987) and contribute significantly to overall respiration (Foissner, 1992). Heterotrophic nanoflagellates belonging to the *Kinetoplastea* and the *Chrysophyceae* occur in a wide range of different environments (Patterson and Lee, 2000; Boenigk et al., 2005) and are also among the most common and abundant groups in soil (Ekelund et al., 2001; Domonell et al., 2013). Analysis of 18S rRNA gene sequences has recently resulted in a new classification of the class *Kinetoplastea*, which includes now the two subclasses *Prokinetoplastina* and *Metakinetoplastina* (Moreira et al., 2004). The *Chrysophyceae* comprise a large group of stramenopiles, including colourless heterotrophic and chloroplast containing flagellates

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(Andersen et al., 1999). Several genera such as the *Spumella*-like morphotype are of urgent need for a revised classification (Boenigk et al., 2005; Boenigk, 2008). Culture-dependent investigations have indicated that soil-borne organisms of the *Spumella* morphotype form 18S rRNA gene clusters separate from their aquatic counterparts. However, both the *Kinetoplastea* and the *Chrysophyceae* have not been addressed so far in terrestrial systems using culture-independent approaches.

Increasing our knowledge of the diversity and overall distribution of the *Kinetoplastea* and the *Chrysophyceae* requires primer sets for the specific amplification of 18S rRNA genes (or gene fragments). To maximise the coverage within the respective target groups and to achieve the highest possible specificity, we chose to develop primer sets targeting an 18S rRNA gene region for which most sequence information of both protist groups was available in public databases. A semi-nested PCR-protocol was established to generate amplification products also from samples with highly fluctuating and low numbers of the *Kinetoplastea* as recently reported (Domonell et al., 2013). Further, we made sure to obtain fragment sizes, which would be suitable for subsequent analysis of the amplification products via terminal restriction fragment length polymorphism (T-RFLP). To this end, suitable restriction enzymes were selected after an *in silico* restriction analysis of soil clone and database sequences.

## 2. Experimental procedures

### 2.1. Primer design

Primer design was based on two 18S rRNA gene sequence databases downloaded in 2009 (re-evaluated in 2012) from the Taxonomy server of GenBank for *Kinetoplastea* (consisting of 206 sequences) and *Chrysophyceae* sensu stricto (208 sequences) (Andersen et al., 1999), respectively. These sequences included also sequences derived from whole genome sequence data. Andersen et al. (1999) showed that the *Synurophyceae* appeared to be embedded within the *Chrysophyceae*, though with only weak bootstrap support. Based thereupon and for the sake of clarity, both classes of the stramenopiles were regarded as one target group termed "*Chrysophyceae*" in the recent study. Potential hairpin-, homo- or heterodimer formations were checked by using the online tool OligoAnalyzer 3.1 (Integrated DNA Technologies, http://eu.idtdna.com). The specificity of the primer was initially evaluated *in silico* by using the platform probeCheck (Loy et al., 2008) of the SILVA database 102 (release 2010) (Pruesse et al., 2007) and BLAST of GenBank (Altschul et al., 1997). Finally two sets of primers for the selected target groups were found to be specific and suitable for PCR-application (Table 1).

PCR conditions for the specific primers were optimised using reference strains and genomic DNA extracted with the PowerSoil DNA extraction kit (MoBio Laboratories, Carlsbad, Canada). PCR was performed in a thermocycler using 2-fold concentrated PCR-Mastermix (Qiagen, Hilden, Germany) and 0.2 μmol of each primer. PCR conditions for both primer pairs consisted of initial denaturation for 3 min at 95 °C, cyclic repeated denaturation for 45 seconds at 95 °C, annealing for 30 seconds at 58 °C and elongation for 70 seconds at 72 °C followed by a final elongation of 5 minutes at 72 °C. Thirty-five PCR cycles were run with the *Chrysophyceae*-specific primer pair Chryso_240/Chryso_651. Semi-nested PCR targeting the *Kinetoplastea* included a first round of PCR (20 cycles) with the universal eukaryotic forward primer Euk20f (Euringer and Lueders, 2008) and the *Kinetoplastea*-specific reverse primer (Kineto_651). A 1:10 dilution of this amplification product was used as a template in a second PCR (30 cycles) with the *Kinetoplastea*-specific primer pair Kineto_80/Kineto_651.

**Table 1**
Primers designed in this study.

| Primer* | Orientation | Sequence (5′-3′) | Target group |
|---|---|---|---|
| Kineto_80 | Forward | CATCAGACGYAATCTGCCGC | *Kinetoplastea* |
| Kineto_651 | Reverse | TTGGTCGCRCTTYTTTAGTCACAG | |
| Chryso_240 | Forward | GGAAACCAATGCGGGGCAAC | *Chrysophyceae* |
| Chryso_651 | Reverse | CTATTTTGCTCACAGTAAATGACGAG | |

* Position refers to *E. coli*.

### 2.2. Cloning and sequencing

Specificity of the primers was further tested by generating a clone library with DNA extracted from soil samples collected in April 2008. The samples originate from two grasslands sites of the German Biodiversity Exploratories (Fischer et al., 2010): Hainich (plot H7), a low-mountain site used as pasture and Schorfheide-Chorin (plot S5), a fen used as mown-pasture. Selected soil parameters of the sites are given in Supplementary Table 1. Within each plot covering an area of 20 m × 20 m, five soil cores were sampled in the corners and the centre, pooled and immediately frozen at −20 and −80 °C. Soil PCR products were checked by agarose gel electrophoresis, purified with the Wizard®SV Gel and the PCR Clean-Up System (Promega Corporation, Madison, USA) and eluted in 30 μl nuclease-free water. The quantity of the purified PCR product was estimated by gel quantification using GeneTools (v 7.01, Syngene Ltd., Parkville, Australia). The PCR product was transformed into *Escherichia coli* DH5α using the pCR®2.1 vector (Invitrogen, Darmstadt, Germany) for subsequent selection of recombinants. DNA of the clones was extracted by transferring biomass into 50 μl water, heating in a microwave (650 W for 45 s) and immediate cooling followed by centrifugation for 10 min at 4 °C. The supernatant was used as template for standard vector PCR amplification using vector specific M13-primers. Product size was checked on a 1.5% agarose gel. PCR products were screened by enzymatic digestion with 2 units MseI (NEB, Ipswich, USA) for the *Kinetoplastea* and HpyCH4III + Sau96I (NEB; each 2 units) for the *Chrysophyceae* at 37 °C overnight. Digested PCR amplicons were run on a 2% agarose gel stained with SyBr® Safe DNA Gel stain (Invitrogen) and scanned using a FLA3000 (Fujifilm, Düsseldorf, Germany). Gel images were analysed using the software Phoretix (PhoretixTM 1D Advanced v5.20; Database v2.00, Nonlinear Dynamics Ltd., Durham, USA).

Purified PCR products from clones (120 in total) were selected according to the restriction patterns (up to five per pattern) and subsequently sequenced. Sequences were determined by Sanger sequencing with the ABI Prism 3130xl genetic analyser (Applied Biosystems, Foster City, USA) using the Big Dye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) and the M13f-primer [0.5 μM]. After manual quality check with the Software Sequencher™ (GeneCodes, USA, version 4.8), sequences were subjected to BLAST searches (Altschul et al., 1997), checked for the presence of chimeras with the software Mallard (Ashelford et al., 2006). Parsimony trees were calculated by means of the programme package ARB (Ludwig, 2004) and added to the Silva small subunit ribosomal RNA reference database version 108 (Pruesse et al., 2007). Sequences were deposited in GenBank under the accession numbers KF188077-KF188194.

We inferred the novelty of the environmental datasets as recently described (del Campo and Massana, 2011). For each sequence we noted its similarity in a BLAST search with the closest environmental match (CEM) and the closest cultured match (CCM). Sequences with high CEM values have been found in other environmental surveys, while sequences with high CCM values highlight sequences with a cultured counterpart. The "novelty degree" of the dataset is obtained by averaging the similarity values for all sequences.

### 2.3. T-RFLP analysis

To identify restriction enzymes with the highest resolution power, we used the online tool Restriction Enzyme Picker (REPK)