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Cultivation-independent analysis reveals a shift in ciliate 18S rRNA gene diversity in a polycyclic aromatic hydrocarbon-polluted soil

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Abstract

Using cultivation-independent methods the ciliate communities of a clay-rich soil with a 90-year record of pollution by polycyclic aromatic hydrocarbons (PAH) (4.5 g kg^{-1} PAH) were compared with that of a nonpolluted soil collected in its vicinity and with similar properties. A ciliate-specific set of 18S rRNA gene targeting primers was designed and used to amplify DNA extracted from both soils (surface and 20 cm depth). Four clone libraries were generated with PCR products that covered an 18S rRNA gene fragment of up to 670 bp. Comparative sequence analysis of representative clones proved that the primer set was highly specific for ciliates. Calculation of similarity indices based on operational taxonomic units after amplified ribosomal DNA restriction analysis of the clones showed that the community from the nonpolluted surface soil was highly dissimilar to the other communities. The presence of several taxa, namely sequences affiliated to the orders *Phyllopharyngia*, *Haptoria*, *Nassophorea*, *Peniculida* and *Scuticociliatia* in samples from nonpolluted soil, points to the existence of various trophic functional groups. In contrast, the 18S rRNA gene diversity was much lower in the clone libraries from the polluted soil. More than 90% of these sequences belonged to the class *Colpodea*, a well-known clade of mainly bacterivorous and r-selected species, thus potentially also indicating a lower functional diversity.

Keywords
ciliates; 18S rRNA genes; PAH; soil protists; microbial eukaryotes.

Introduction

During the last two decades, the use of molecular methods significantly increased our knowledge of prokaryotic diversity and ecology in environmental systems (Woese, 1987). Until recently, studies on the biodiversity of unicellular eukaryotic organisms mainly relied on microscopic observation and culture-dependent description based on morphological criteria. However, in particular, small protists such as soil flagellates are difficult to observe by direct microscopic examination of environmental samples. Direct microscopic observation of larger protists, e.g. ciliates and testate amoebae seems to be feasible, and yet, a proper and reliable community description requires highly skilled taxonomists and appropriate identification literature. Ciliate identification, for instance, is currently performed using a combination of different microscopic techniques on living and fixed cells and often time-consuming staining techniques to visualize morphological structures (Foissner, 1999). Observation of ciliate populations in fresh samples may be hampered by the fact that e.g. many ciliates are dying within a short time when observed under a cover slip, whereas fixation of environmental samples often results in drastic changes of cell shapes (Choi & Stoecker, 1989).

Unlike for prokaryotic communities, rRNA based techniques have only recently been 'discovered' for the study of natural protistan communities in soil, marine or freshwater systems (Lopez-Garcia *et al.*, 2001; Lawley *et al.*, 2004; Richards *et al.*, 2005). First attempts to screen protistan communities involved FISH with 18S rRNA gene targeting domain-specific (Lim *et al.*, 1996) or species-specific probes (e.g. Rice *et al.*, 1997). Later studies involved fingerprinting techniques such as denaturing gradient gel electrophoresis to monitor mostly aquatic eukaryotic communities (Diez

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et al., 2001) and environmental 18S rRNA gene clone libraries that provided new insights into the diversity of marine and freshwater picoeukaryotes (Lopez-Garcia *et al.*, 2001; Richards *et al.*, 2005). The authors of several studies came to the conclusion that still-undescribed protistan diversity exists in these ecosystems and that, similar to the prokaryotes, a significant part of protists may have escaped detection by conventional methods (e.g. Lopez-Garcia *et al.*, 2001; Richards *et al.*, 2005; Šlapeta *et al.*, 2005).

Only a few studies extended the application of rRNA-based methods to microbial eukaryotic communities in soil (Anderson & Cairney, 2004; Lawley *et al.*, 2004; Fell *et al.*, 2006; Moon-van der Staay *et al.*, 2006). In addition, because in most of the culture-independent molecular analyses only universal eukaryotic primers were used, it has been suggested that the use of several combinations of universal or group-specific primers might allow for a more comprehensive picture of the microeukaryotic diversity and species richness in environmental systems (Stoeck *et al.*, 2006). Protists are a polyphyletic group, thus making the design of specific rRNA gene targeting primers for various protistan taxa necessary. So far, the authors are aware of only a few environmental studies that are not related to public health issues and that apply primers specific for distinct soil protistan groups, such as the kinetoplastida and the cercozoa (Rasmussen *et al.*, 2001; Bass & Cavalier-Smith, 2004).

Among the traditional functional groups of soil protists that ecologists distinguish (flagellates, testate amoebae, naked amoebae and ciliates), ciliates or the *Ciliophora* are accepted as the only monophyletic assemblage. 18S rRNA gene sequence analysis confirmed this monophyly, and identified apicomplexans and dinoflagellates as the sister group to ciliates (Gajadhar *et al.*, 1991). Ciliates are an important and diverse group of soil-inhabiting protists: about 800 species have been described in soil and their number is still increasing (Foissner *et al.*, 2002). The ecological requirements of these organisms are very diverse: most feed on bacteria, some are predaceous on other protists whereas others are strictly mycophagous. Anaerobic ciliates belonging to different taxa have been described using culture-based but also cultivation-independent approaches (Fenchel & Finlay, 1995; Takishita *et al.*, 2007). Reproductive strategies of ciliates in soils have also been studied, and while some taxa (e.g. members of the *Colpodea*) have explosive growth rates due to a special reproductive strategy by quadripartition in the cysts and are highly competitive in disturbed soils (r-strategists), others (such as the *Spirotrichea*) have longer generation times and are found in more stable environments (K-strategists) (Lüftenegger *et al.*, 1985). The sensitivity of ciliates to a wide palette of toxicants has been extensively studied in model systems (Sauvant *et al.*, 1999 and references therein) as well as in the field (Petz & Foissner, 1989). Therefore, they potentially represent a group of excellent bioindicator organisms for the effects of a pollutant on an environmental system (Foissner, 1999). However, there is a need for new tools accessible both to protistologists and to nonspecialists to study the distribution, diversity and ecology of soil ciliates and to evaluate the use of these organisms as bioindicators.

Here, the results are reported of a molecular survey of the ciliate 18S rRNA gene pool in a soil exposed for several decades to a mixture of polycyclic aromatic hydrocarbons (PAH) and a nonpolluted soil. A new set of PCR primers was used for the specific amplification of partial ciliate 18S rRNA gene sequences and the subsequent creation of environmental clone libraries from the soil surface and from 20 cm depth.

Materials and methods

Sample collection

Samples were taken from a PAH-contaminated site at the former railway station in Andújar (Province of Jaén, Spain). Soil analysis was performed as described in Niqui-Arroyo *et al.* (2006). The PAH contamination is a result of the treatment of the wooden railway sleepers with creosote during the last 90 years. The soil is a silty clay (3.4% sand, 37% silt and 59.5% clay) and overlies a water-bearing horizon of sandy gravel. The polluted soil samples had a total PAH content of 4.5 g kg^{-1} dry soil. Samples were also taken outside the contaminated area to obtain a nonpolluted soil (1.4% sand, 31.1% silt, 67.4% clay). In order to account for spatial heterogeneity, samples were taken from the nonpolluted soil (1) at the surface (< 5 cm) at 1–6 m distance from each other ($n = 4$) and (2) at 20 cm depth at 0.5 m distance from each other ($n = 3$). Two samples at the surface and five samples at 20 cm depth were taken from the polluted soil at 0.5 m distance from each other. All sampling was performed using an auger, whose head was changed after each sampling in order to avoid cross-contamination.

Total soil community DNA extraction

A quantity of 0.6 g of soil from each sampling point was vortexed in a 50-mL sterile Falcon tube with 1.2 mL phosphate buffer (0.1 M Na_3PO_4, pH 8) until the clay was completely suspended. After the addition of 0.6 mL lysis buffer (0.1 M NaCl; 0.5 M Tris, pH 8.0; 10% sodium dodecyl sulfate), the suspension was distributed into three cryotubes. Glass beads (0.5 g) as well as 0.5 mL phenol/chloroform/isoamylalcohol (25 : 24 : 1) were added. The tubes were shaken for 45 s in a FastPrep bead beater (Bio 101) at 5.5 m s^{-1} and subsequently centrifuged for 5 min at 16 100 ***g***. The aqueous phase was then removed and DNA was precipitated with two volumes of 30% polyethylene

Table 1. PCR primers used for the specific amplification of ciliate 18S rRNA gene fragments

Primer	Sequence 5′–3′	Position
Cil-f	TGGTAGTGTATTGGACWACCA	315
Cil-r I	TCTGATCGTCTTTGATCCCTTA	959
Cil-r II	TCTRATCGTCTTTGATCCCCTA	959
Cil-r III	TCTGATTGTCTTTGATCCCCTA	959

Cil-f indicates the forward primer and Cil-r I, II and III the reverse primers. Positions are given in reference to *Tetrahymena australis* (X56167).

glycol 6000/1.6 M NaCl for 2 h at room temperature, followed by centrifugation (10 min at 16 100 ***g***). Pelleted nucleic acids were washed in 70% ethanol and air-dried before resuspension in 20 µL sterile water. Resuspended DNA from the same sample was then pooled in a single tube.

Primer design

In order to design ciliate-specific primers, 154 ciliate 18S rRNA gene sequences from representatives of all ciliate classes were retrieved from GenBank (National Centre for Biotechnology Information) and aligned using the 'CLUSTAL W' option (Thompson *et al.*, 1997) in the BIOEDIT software (Hall, 1999) with default parameters. One forward primer and three reverse primers (on the same target position in the gene) were designed (Table 1) covering a 600–670-bp fragment. The difference in size is due to small insertions spread all over the ciliate 18S rRNA gene. BLAST search in GenBank was used to evaluate the specificity of these primers on all available nuclear ciliate 18S rRNA gene sequences.

PCR amplification and clone library construction

Total DNA extracts from soil samples were diluted 20 times in sterile water before PCR amplification. The PCR mixtures (25 µL) contained 0.2 µM of the forward primer, each deoxynucleoside triphosphate at a concentration of 200 µM, 2 mM $MgCl_2$, 1.25 U of Taq DNA polymerase (Qiagen), 3.6 mg mL^{-1} acetylated bovine serum albumin (BSA) and the 10 × PCR buffer supplied with the enzyme. The reverse primer (0.2 µM) consisted of a mixture of the three reverse primers Cil-r I, II and III. Reactions were carried out in a PTC 200 thermocycler (MJ Research) using the following programme: an initial 5-min denaturation at 95 °C, followed by 35 cycles of denaturation at 94 °C for 45 s, annealing at 58 °C for 1 min, elongation at 72 °C for 1 min and a final extension step of 10 min at 72 °C. All amplification products from replicate DNA templates originating from the same depth were pooled and subsequently used for cloning. The pooled PCR products were used to produce four clone libraries, named PS05 (polluted soil, surface samples), PS20 (polluted soil, 20 cm depth samples), NPS05 (nonpolluted soil, surface samples) and NPS20 (nonpolluted soil, 20 cm depth samples). They were cloned into the vector (pCR® II-TOPO®) supplied with an HTP TOPO TA Cloning® Kit Dual Promoter (Promega) by following the manufacturer's recommendations. Putative positive colonies were picked and directly amplified using the vector primers T7 and SP6. PCR products were checked on an agarose gel. Ten microliters of the PCR products were digested with the restriction enzyme Taq I (Promega) following the manufacturer's recommendations. The digests were run on a 3% Metaphor (BMA) low-melting gel at 110 V for 75 min. For each digestion pattern, at least four clones were chosen and sequenced in order to check the resolution power of the amplified ribosomal DNA restriction analysis (ARDRA).

Sequencing and phylogenetic analysis

Sequencing reactions were performed with an ABI Prism® Big Dye™ Terminator v 3.0 Ready Reaction Cycle Sequencing Kit (Applied Biosciences) and an ABI PRISM model 3100 automated sequencer. Sequences were subjected to a BLAST search in GenBank to determine the most closely related sequence in the database. The presence of potentially chimeric sequences was checked by identifying 'group-specific signature sequences' and by partial treeing analyses as recommended by Berney *et al.* (2004). The 18S rRNA gene sequences obtained in this study were aligned manually using the BIOEDIT software (Hall, 1999), following the secondary structure model proposed by Wuyts *et al.* (2000). Additional sequences from public databases were selected to illustrate optimally the phylogenetic position of the sequences. Because of the higher divergence of the 18S rRNA gene in some ciliate classes (in particular the *Phyllopharyngea*), two datasets were constructed and analysed separately, in order to avoid losing too much resolution within other classes (in particular, the *Oligohymenophorea*). The number of unambiguously aligned positions that were used in the phylogenetic analyses was 562 for the first dataset (classes *Litostomatea*, *Phyllopharyngea* and *Spirotrichea*) and 625 for the second dataset (classes *Colpodea*, *Nassophorea* and *Oligohymenophorea*). All phylogenetic analyses were performed using the GTR model of substitution (Rodriguez *et al.*, 1990), taking into account a proportion of invariable sites, and a γ-shaped distribution of the rates of substitution among variable sites, with eight rate categories. All necessary parameters were estimated from the datasets. For each dataset, a minimum evolution phylogenetic tree was inferred with maximum likelihood-corrected estimates of the pairwise distances between sequences, using the BioNJ option in PAUP* (Swofford, 1998). The reliability of internal branches was assessed with the bootstrap method (Felsenstein, 1985), with 10 000 replicates. In addition, Bayesian

analyses were performed with MrBayes (Huelsenbeck & Ronquist, 2001). For each dataset, four simultaneous chains were run for 1 200 000 generations, and 12 000 trees were sampled, 2000 of which were discarded as the burn-in. Posterior probabilities at all nodes were estimated from the 10 000 remaining trees. The sequences have been deposited in the GenBank database under the accession numbers DQ115936–DQ115966. Statistical analysis and comparison of ciliate community structures in the clone libraries were performed with the program SONS (Schloss & Handelsman, 2006). The abundance-based Jacard (J_{abund}) has been described as a measure of community overlap, which is defined as the probability that a randomly selected operational taxonomic units (OTU) is found in both communities, given that it is in at least one of the communities. Because J_{abund} does not account for the similarity of the relative abundances among the OTUs shared between two communities, community structure similarity was estimated with a nonparametric maximum likelihood estimator θ (Schloss & Handelsman, 2006). Rarefaction calculations as a measure of coverage of the clone libraries were performed using the software ANALYTIC RAREFACTION (version 1.3; http://www.uga.edu/~strata/software).

Results and discussion

Primer design and establishment of clone libraries

Based on an alignment of all ciliate sequences retrieved from GenBank (National Center for Biotechnology Information), the primer pair Cil-f and Cil-r was designed to amplify partial 18S rRNA gene sequences of ciliates (Table 1). Depending on the ciliate taxon, a 600–670-bp fragment is covered by these primers. The size variance is due to small insertions spread all over the 18S rRNA gene found in particular within members of the genera *Euplotes*, *Euplotoides* and *Moneuplotes* (family *Euplotidae*). Major deletions occur in the variable V4 region of litostomatean ciliates. A BLAST search in GenBank had shown that Cil-f perfectly matched with the target site of all available ciliate 18S rRNA gene sequences. Additional hits were obtained for rRNA gene sequences from Fungi, Metazoa and Stramenopiles (mainly the diatoms and the genus *Blastocystis*), whereas some 18S rRNA gene sequences from Fungi, Metazoa, Stramenopiles and one Cercozoa had only one mismatch in the center of the target sequence. The reverse primer Cil-r is a combination of three primers in order to cover mismatches found in the 18S rRNA gene sequences of several ciliate taxa, in particular, from members of the more divergent *Litostomatea*, *Phyllopharyngea*, *Peritrichia*, *Heterotrichea* and *Karyorelictea*. Several fungal and stramenopile rRNA gene sequences had the same target sequence or showed only one mismatch. However, in none of these cases did a nontarget organism show a perfect match or only one mismatch in both target sites. It was therefore concluded that the rRNA gene sequences from these nontarget organisms would not be amplifiable.

Four clone libraries were generated, designated PS05 (polluted, surface samples), PS20 (polluted, 20 cm depth samples), NPS05 (nonpolluted, surface samples) and NPS20 (nonpolluted, 20 cm depth samples). These libraries contained 125, 85, 153 and 75 clones, respectively. For each distinct ARDRA pattern (treated as OTU), up to four clones were sequenced. Some clones showing identical ARDRA pattern corresponded to sequences that were not closely related to each other. This was the case for the clones representing OTU 4; all of these clones were therefore sequenced and could be affiliated to at least 10 different ciliate genera (Table 2). In general, within-OTU sequence similarity was between 96% and 100%.

Comparative phylogenetic analysis of the clone libraries

Nine clones were excluded from further analysis due to their potentially chimerical nature. BLAST analysis of the remaining 93 18S rRNA gene sequences showed that all sequences represented only organisms within the ciliates, thus demonstrating the specificity of the primer set. The overall affiliation of the sequenced clones to different ciliate classes and the relative abundance of these classes within the clone libraries are summarized in Fig. 1, whereas the nearest database relative obtained by BLAST search is shown in Table 2. Thirty-one sequences representing the different OTU are shown in the corresponding phylogenetic trees in Fig. 2a and b. Overall, the sequences clustered within six ciliate classes, i.e. the *Phyllopharyngea*, the *Colpodea*, the *Oligohymenophorea*, the *Nassophorea*, the *Spirotrichea* and the *Litostomatea*. Most ciliate taxa appeared monophyletic in the phylogenetic tree, despite the small size of the 18S rRNA gene fragment analysed and were supported by both bootstrap and Bayesian analysis, except for the class *Nassophorea* and the subclass *Scuticociliata*, the monophyly of the latter having been anyway considered as doubtful (Song *et al.*, 2005). A substantial number of the sequences are representative of the most common ciliate taxa found in soils, such as the *Colpodea*, *Spirotrichea* and *Haptoria*, while the other groups have been described as being at least present in soils (Foissner, 1998). Clone libraries from polluted soil were dominated by sequences affiliating to the class *Colpodea*, which accounted for 90% and 98%, respectively, of all analysed clones in each library (Fig. 1, Table 2). In contrast to this, the diversity and distribution of ciliate taxa were different in the clone libraries originating from nonpolluted

Table 2. List of all OTUs obtained, with taxonomic affiliation and number of clones per clone library

Class	Order	OTU	Closest relative (sequence identity)	PS05	PS20	NPS05	NPS20	Total PS	Total NPS	Total 5 cm	Total 20 cm
Phyllopharyngea											
	Phyllopharyngia	1	Uncultured alveolate clone LEMD069 (98%)	0	0	23	6	29	0	23	6
		1	Uncultured alveolate clone LEMD069 (90%)	0	0	0	1	1	0	0	1
	Suctoria	4	*Heliophrya erhardi (90%)*	0	0	2	0	2	0	2	0
		4	*Acineta* sp. OSW-2003-1 (85%)	3	0	0	0	0	3	3	0
		4	*Ephelota* sp. RJL2001 (85%)	1	0	0	0	0	1	1	0
Oligohymenophorea											
	Peniculida	2	*Frontonia* sp. (91%)	0	0	63	0	63	0	63	0
	Peritrichia	3	*Opisthonecta henneguyi* (98%)	2	0	3	0	3	2	5	0
	Hymenostomatida	17	*Ophryoglena catenula* (94%)	0	0	0	2	2	0	0	2
	Scuticociliatia	8	Uncultured ciliate clone CCW108 (96%)	0	0	7	1	8	0	7	1
		4	*Parauronema longum* (91%)	0	0	0	1	1	0	0	1
		10	*Dexitrichides pangi* (91%)	0	0	3	0	3	0	3	0
Nassophorea											
	Nassophorea incertae sedis	19	*Pseudomicrothorax dubius* (89%)	0	0	0	1	1	0	0	1
		16	*Obertrumia georgiana* (91%)	0	0	0	1	1	0	0	1
Spirotrichia											
	Stichotrichia	15	*Oxytricha granulifera* (98%)	1	0	0	4	4	1	1	4
		4	*Oxytricha longa* (100%)	1	0	0	0	0	1	1	0
		4	*Oxytricha trifallax* (99%)	0	0	0	2	2	0	0	2
		4	*Pattersoniella vitiphila* (100%)	3	0	0	1	1	4	4	1
		4	*Gastrostyla steini* (100%)	0	1	0	1	1	0	0	1
		4	*Gonostomum strenuum* (99%)	0	1	0	1	1	1	0	2
	Oligotrichia	4	*Halteria grandinella* (98%)	0	0	7	0	7	0	7	0
		4	*Halteria grandinella* (94%)	0	1	0	3	3	1	0	4
Litostomatea											
	Haptoria	14	*Spathidium* sp. (97%)	0	0	3	6	9	0	3	6
		11	*Spathidium* sp. (95%)	0	0	10	1	11	0	10	1
Colpodea											
	Cyrtolophosida	9	*Platyophrya vorax* (99%)	6	0	1	1	2	6	7	1
	Colpodida	12	*Bresslaua vorax* (97%)	4	1	3	12	15	5	7	13
		13	*Bresslaua vorax* (98%)	13	7	0	0	0	20	13	7
		6	*Pseudoplatyophrya nana* (98%)	35	39	9	11	20	74	44	50
		5	*Pseudoplatyophrya nana* (97%)	43	5	15	12	27	48	58	17
		7	*Pseudoplatyophrya nana* (96%)	12	27	2	4	6	39	14	31
	Colpodea incertae sedis	4	LKM 63 (93%)	1	3	1	3	4	4	2	6
		18	LKM 63 (91%)	0	0	1	0	1	0	1	0
			Total	125	85	153	75	228	210	279	159

PS05, clone library derived from the polluted soil, surface 5 cm depth; PS20, clone library derived from the polluted soil, 20 cm depth; NPS05, clone library derived from the nonpolluted soil, surface; NPS20, clone library derived from the nonpolluted soil, 20 cm depth.

soil (Fig. 1, Table 2). The NPS05 clone library was dominated by sequences affiliated to the *Oligohymenophorea* (76), *Colpodea* (32) and Phyllopharyngea (25), whereas the NPS20 clone library mainly consisted of sequences affiliated to the *Colpodea* (43), *Spirotrichea* (12), *Phyllopharyngia* (7) and *Litostomatea* (7). Although the abundance of OTUs in clone libraries does not necessarily reflect the true abundance of protists in an ecosystem due to PCR-associated biases (von Winzingerode *et al.*, 1997) and varying rRNA gene copy numbers (Ward *et al.*, 1997), the overlap between the communities on the OTU level as observed in the clone libraries was further estimated: the fraction of the sequences in the clone libraries from the polluted soil that belonged to shared OTUs was between 80% and 98%, whereas only 19–49% of the total number of sequences in NPS05 was shared with the other libraries. The abundance-based Jaccard (J_{abund}) index showed high community overlap between PS05, PS20 and NPS20. The J_{abund} values were large (0.79–0.87), suggesting that although low abundance members might not be shared between the communities, the most abundant were shared. The high values obtained for these communities indicate that the living conditions in the deeper layers might be as harsh as in polluted soils and may therefore favour the survival of similar organisms. In

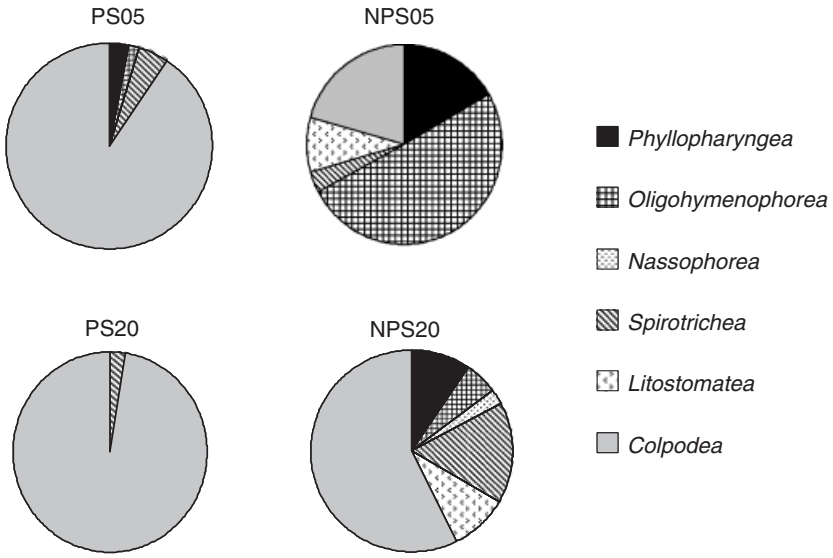
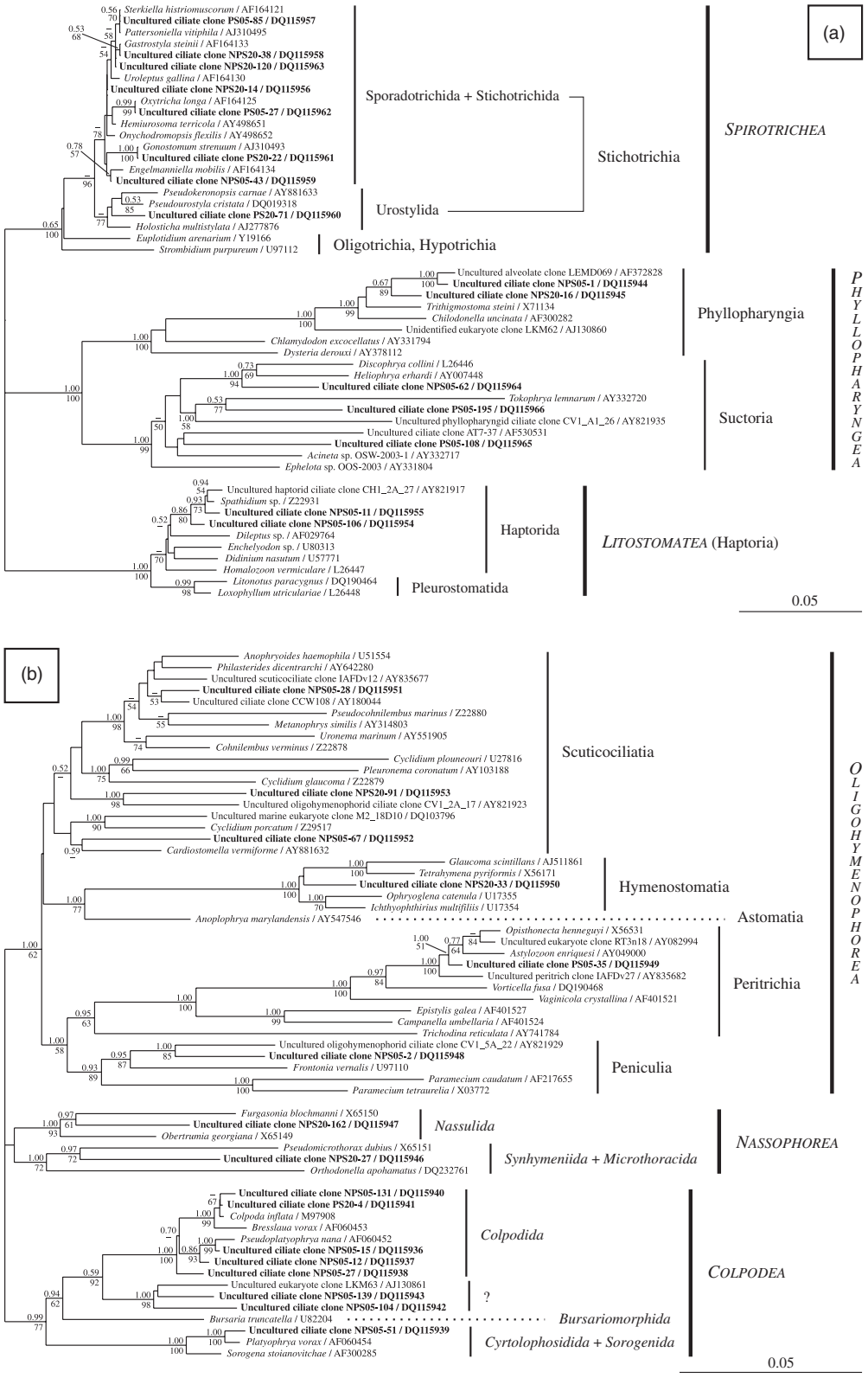


Fig. 1. Charts representing the class-level taxonomic composition of clone libraries PS05 (polluted soil, surface), PS20 (polluted soil, 20 cm), NPS05 (nonpolluted soil, surface) and NPS20 (nonpolluted soil, 20 cm). Taxonomic affiliation of the 18S rRNA gene sequences is based on phylogenetic analysis as described in 'Materials and methods'.

contrast to this, the J_{abund} between NPS05 and PS05, PS20 or NPS20 was much lower (0.21, 0.2 and 0.54, respectively). However, because the overlap measure J_{abund} does not account for the similarity of the relative abundances among the OTUs shared between two communities, the community similarity index θ was calculated (Schloss & Handelsman, 2006). The θ values for the comparisons between PS05 and PS20, PS05 and NPS20 and PS20 and NPS20 were 0.52, 0.52 and 0.3, respectively. Comparing NPS05 with all the other clone libraries yielded values between 0.07 and 0.16. Rarefaction analysis was performed to estimate to what extent the diversity of the samples could be described with the number of clones analysed. Flattening accumulation curves were observed for all samples (most pronounced for NPS05), with the exception of NPS20, indicating that the number of clones was insufficient to describe in particular the diversity in NPS20 (Fig. 3). Therefore, the calculated values for J_{abund} and the community similarity index θ are only an estimation and will probably change with additional sampling.

The ciliate 18S rRNA gene diversity from the polluted soil was in general characterized by a reduction of diversity. The results show that the PAH contamination clearly affected the 18S rRNA gene diversity of the ciliate community, either by its direct toxic effects, or by its effects on the soil food web, i.e. the ciliates' prey. Preliminary direct observation of enrichment cultures showed that *Colpodea* are also dominating the (cultivable) ciliate community in the polluted soil (data not shown). *Colpodea* are typically bacterivorous ciliates (Foissner, 1998); however, one exception worth mentioning is *Pseudoplatyophrya nana*, which is specialized to prey upon fungi. As potential r-strategists, *Colpodea* are expected to cope better with xenobiotic pollution or stress than K-selected taxa. Petz & Foissner (1989), for instance, demonstrated that the application of xenobiotics such as the insecticide lindane selected for *Colpodea* (*Colpoda inflata*, *Colpoda steinii*, *Pseudoplatyophrya nana*), whereas more sensitive taxa were eliminated. Most of the *Colpodea* sequences fall into the order *Colpodida* and were closely related to already described species, i.e. *Bresslaua vorax*, *Colpoda inflata* and the mycophagous *Pseudoplatyophrya nana* (Fig. 2b). In addition to the *Colpodida*, six *Cyrtolophosida* closely related to *Platyophrya vorax* and four sequences related to the environmental clone sequence LKM 63 were identified in the clone library from polluted soil (Snoeyenbos-West *et al*., 2004). The latter group appears to be a sister group to the *Colpodida*, potentially forming a novel colpodean clade, and is supported by a high bootstrap value. Of the remaining ($n = 14$), noncolpodean 18S rRNA gene sequences detected in the polluted soil samples, two clones clustered with the peritrichs *Opisthonecta henneguyi* and *Astylozoon enriquesi*, eight were spirotrichs and four

Fig. 2. Minimum evolution trees (GTR+G+I model) showing the phylogenetic position of the 31 representative 18S rRNA gene sequences. The numbers at nodes indicate the posterior probabilities as calculated with Bayesian analyses (above) and the bootstrap support values after 10 000 replicates (below). Values under 0.5/50% were omitted. All branches are drawn to the corresponding scale. Both trees are presented in an unrooted format, with a basal trifurcation. (a) Evolutionary relationships within classes *Litostomatea*, *Phyllopharyngea* and *Spirotrichea* (proportion of invariable sites: 10.98%; parameter α: 0.3094). (b) Evolutionary relationships within classes *Colpodea*, *Nassophorea* and *Oligohymenophorea* (proportion of invariable sites: 11.37%; parameter α: 0.2884).


(a)
Sterkiella histriomuscorum / AF164121
Uncultured ciliate clone PS05-85 / DQ115957
Pattersoniella vitiphila / AJ310495
Gastrostyla steinii / AF164133
Uncultured ciliate clone NPS20-38 / DQ115958
Uncultured ciliate clone NPS20-120 / DQ115963
Uroleptus gallina / AF164130
Uncultured ciliate clone NPS20-14 / DQ115956
Oxytricha longa / AF164125
Uncultured ciliate clone PS05-27 / DQ115962
Hemiurosoma terricola / AY498651
Onychodromopsis flexilis / AY498652
Gonostomum strenuum / AJ310493
Uncultured ciliate clone PS20-22 / DQ115961
Engelmanniella mobilis / AF164134
Uncultured ciliate clone NPS05-43 / DQ115959
Pseudokeronopsis carnae / AY881633
Pseudourostyla cristata / DQ019318
Uncultured ciliate clone PS20-71 / DQ115960
Holosticha multistylata / AJ277876
Euplotidium arenarium / Y19166
Strombidium purpureum / U97112
Sporadotrichida + Stichotrichida
Stichotrichia
Urostylida
Oligotrichia, Hypotrichia
SPIROTRICHEA
Uncultured alveolate clone LEMD069 / AF372828
Uncultured ciliate clone NPS05-1 / DQ115944
Uncultured ciliate clone NPS20-16 / DQ115945
Trithigmostoma steini / X71134
Chilodonella uncinata / AF300282
Unidentified eukaryote clone LKM62 / AJ130860
Chlamydodon excocellatus / AY331794
Dysteria derouxi / AY378112
Phyllopharyngia
Discophrya collini / L26446
Heliophrya erhardi / AY007448
Uncultured ciliate clone NPS05-62 / DQ115964
Tokophrya lemnarum / AY332720
Uncultured ciliate clone PS05-195 / DQ115966
Uncultured phyllopharyngid ciliate clone CV1_A1_26 / AY821935
Uncultured ciliate clone AT7-37 / AF530531
Uncultured ciliate clone PS05-108 / DQ115965
Acineta sp. OSW-2003-1 / AY332717
Ephelota sp. OOS-2003 / AY331804
Suctoria
PHYLLOPHARYNGEA
Uncultured haptorid ciliate clone CH1_2A_27 / AY821917
Spathidium sp. / Z22931
Uncultured ciliate clone NPS05-11 / DQ115955
Uncultured ciliate clone NPS05-106 / DQ115954
Dileptus sp. / AF029764
Enchelyodon sp. / U80313
Didinium nasutum / U57771
Homalozoon vermiculare / L26447
Litonotus paracygnus / DQ190464
Loxophyllum utriculariae / L26448
Haptorida
Pleurostomatida
LITOSTOMATEA (Haptoria)
0.05
(b)
Anophryoides haemophila / U51554
Philasterides dicentrarchi / AY642280
Uncultured scuticociliate clone IAFDv12 / AY835677
Uncultured ciliate clone NPS05-28 / DQ115951
Uncultured ciliate clone CCW108 / AY180044
Pseudocohnilembus marinus / Z22880
Metanophrys similis / AY314803
Uronema marinum / AY551905
Cohnilembus verminus / Z22878
Cyclidium plouneouri / U27816
Pleuronema coronatum / AY103188
Cyclidium glaucoma / Z22879
Uncultured ciliate clone NPS20-91 / DQ115953
Uncultured oligohymenophorid ciliate clone CV1_2A_17 / AY821923
Uncultured marine eukaryote clone M2_18D10 / DQ103796
Cyclidium porcatum / Z29517
Uncultured ciliate clone NPS05-67 / DQ115952
Cardiostomella vermiforme / AY881632
Scuticociliatia
Glaucoma scintillans / AJ511861
Tetrahymena pyriformis / X56171
Uncultured ciliate clone NPS20-33 / DQ115950
Ophryoglena catenula / U17355
Ichthyophthirius multifiliis / U17354
Hymenostomatia
Anoplophrya marylandensis / AY547546
Astomatia
Opisthonecta henneguyi / X56531
Uncultured eukaryote clone RT3n18 / AY082994
Astylozoon enriquesi / AY049000
Uncultured ciliate clone PS05-35 / DQ115949
Uncultured peritrich clone IAFDv27 / AY835682
Vorticella fusa / DQ190468
Vaginicola crystallina / AF401521
Peritrichia
Epistylis galea / AF401527
Campanella umbellaria / AF401524
Trichodina reticulata / AY741784
Uncultured oligohymenophorid ciliate clone CV1_5A_22 / AY821929
Uncultured ciliate clone NPS05-2 / DQ115948
Frontonia vernalis / U97110
Paramecium caudatum / AF217655
Paramecium tetraurelia / X03772
Peniculia
OLIGOHYMENOPHOREA
Furgasonia blochmanni / X65150
Uncultured ciliate clone NPS20-162 / DQ115947
Obertrumia georgiana / X65149
Nassulida
Pseudomicrothorax dubius / X65151
Uncultured ciliate clone NPS20-27 / DQ115946
Orthodonella apohamatus / DQ232761
Synhymeniida + Microthoracida
NASSOPHOREA
Uncultured ciliate clone NPS05-131 / DQ115940
Uncultured ciliate clone PS20-4 / DQ115941
Colpoda inflata / M97908
Bresslaua vorax / AF060453
Pseudoplatyophrya nana / AF060452
Uncultured ciliate clone NPS05-15 / DQ115936
Uncultured ciliate clone NPS05-12 / DQ115937
Uncultured ciliate clone NPS05-27 / DQ115938
Colpodida
Uncultured eukaryote clone LKM63 / AJ130861
Uncultured ciliate clone NPS05-139 / DQ115943
Uncultured ciliate clone NPS05-104 / DQ115942
?
Bursaria truncatella / U82204
Bursariomorphida
Uncultured ciliate clone NPS05-51 / DQ115939
Platyophrya vorax / AF060454
Sorogena stoianovitchae / AF300285
Cyrtolophosidida + Sorogenida
COLPODEA
0.05

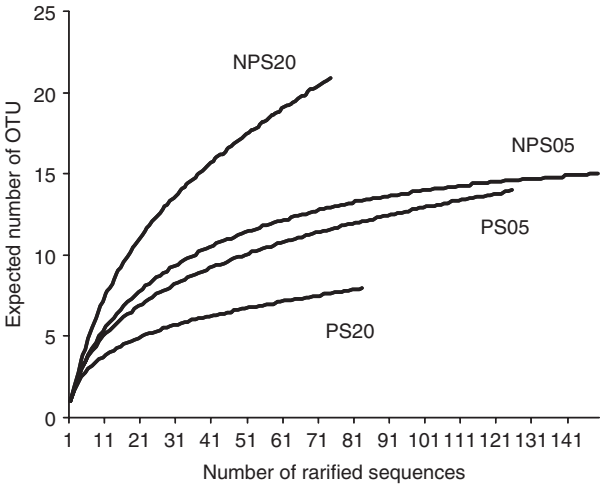


Fig. 3. Rarefaction analysis of all four clone libraries. Distinct sequence types detected among OTU 4 were treated for simplicity reasons as OTUs.

were distantly related to members of the subclass Suctoria (*Acineta* sp. and *Ephelota* sp.).

Sequences branching within the same colpodean and spirotrichean orders and genera were also abundant in the nonpolluted soil clone libraries. However, several ciliate orders and even classes were exclusively found in clone libraries from nonpolluted soil such as the *Phyllopharingia*, *Peniculida*, *Scuticociliatia*, *Nassophorea* and *Haptoria*. Because many cyrtophorids are exclusive diatom predators, *Haptoria* feed only on other protists and all known Nassophoreans prey upon cyanobacteria (Verni & Gualtieri, 1997; Foissner, 1998), one may speculate that similar to taxonomic diversity, functional diversity might be also higher in the nonpolluted soil; the absence of these organisms might well be due to the absence of their preys.

Although ciliates are among the best-studied protistan groups, some of the sequences were only distantly related to described species, especially those belonging to the *Oligohymenophorea*, *Nassophorea* and *Suctoria*. This is in agreement with other environmental molecular surveys and shows that ciliate molecular diversity has by far not yet been completely captured (e.g. Šlapeta *et al.*, 2005; Stoeck *et al.*, 2006). It is nevertheless likely that these organisms have already been described morphologically in other studies.

The value of molecular-based screening tools for soil protists

The aim of this study was to develop a ciliate-specific PCR-protocol and to compare and analyse ciliate 18S rRNA gene sequences from a PAH-polluted and a nonpolluted soil. It has been shown that it is possible to obtain with the used DNA-extraction and ciliate-specific primers a broad spectrum of ciliate 18S rRNA gene sequences covering several taxa, suggesting that a bias against the PCR-amplification of distinct ciliate taxa seems unlikely. Applying specific primers to DNA derived from soil potentially allows obtaining a much wider range of ciliate sequences, because 18S rRNA genes from nonprotistan eukaryotic organisms, such as fungi or metazoans, may dominate soil clone libraries generated with 'universal' eukaryotic primers (Lawley *et al.*, 2004; Moon-van der Staay *et al.*, 2006). Although it could not be distinguished which of the sequences were retrieved from vegetative cells or cysts, it is believed that the application of such a molecular screening tool in addition to classical methods, stable-isotope techniques and statistical tools would also encourage nontaxonomists to study this group of organisms and evaluate their potential use as 'indicators' for stressed environments.

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