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Application of the denaturing gradient gel electrophoresis (DGGE) technique as an efficient diagnostic tool for ciliate communities in soil

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Ciliates (or Ciliophora) are ubiquitous organisms which can be widely used as bioindicators in ecosystems exposed to anthropogenic and industrial influences. The evaluation of the environmental impact on soil ciliate communities with methods relying on morphology-based identification may be hampered by the large number of samples usually required for a statistically supported, reliable conclusion. Cultivation-independent molecular-biological diagnostic tools are a promising alternative to greatly simplify and accelerate such studies. In this present work a ciliate-specific fingerprint method based on the amplification of a phylogenetic marker gene (i.e. the 18S ribosomal RNA gene) with subsequent analysis by denaturing gradient gel electrophoresis (DGGE) was developed and used to monitor community shifts in a polycyclic aromatic hydrocarbon (PAH) polluted soil. The semi-nested approach generated ciliate-specific amplification products from all soil samples and allowed to distinguish community profiles from a PAH-polluted and a non-polluted control soil. Subsequent sequence analysis of excised bands provided evidence that polluted soil samples are dominated by organisms belonging to the class Colpodea. The general DGGE approach presented in this study might thus in principle serve as a fast and reproducible diagnostic tool, complementing and facilitating future ecological and ecotoxicological monitoring of ciliates in polluted habitats.

Keywords: Ciliates, DGGE, 18S rRNA, Soil, Monitoring, Pollution, PAH

1. Introduction

Ciliates are unicellular eukaryotes present in virtually all ecosystems. More than 800 species have been described from terrestrial habitats (Foissner et al., 2002). Their importance as key mediators for several soil ecosystem processes such as the enhancement and regulation of nutrient cycling or decomposition rates to the benefit of plants and microorganisms has been well documented (Clarholm, 1985). Soil ciliate communities comprise several functional groups such as the usually dominating bacterivores, the fungivores, the omnivores and the detritivores. Their ubiquity and their role as an essential component of terrestrial systems, combined with their morphological and biological characteristics, such as fragile external membranes and rapid growth, make them suited as early warning systems and in situ biodindicators (Foissner, 1997, 1999). Several reports have shown that ciliates are susceptible to a wide range of environmental stressors such as insecticides (Petz and Foissner, 1989), biocides (Ekelund, 1999; Foissner, 1997), heavy metals or polychlorinated biphenyls (Foissner, 1994), and are thus a potential target group for screening the ecotoxicological effects of pollutants (Sauvant et al., 1999).

Monitoring stress-induced composition shifts in ciliate communities within multiple environmental samples would be substantially simplified by the application of rapid diagnostic tools which circumvent biases inherent to cultivation-based environmental surveys. These biases include undersampling of habitats, inconsistent resolution of in situ identification and observation, but also lack of suitable cultivation techniques. Recent cultivation-independent surveys based on phylogenetic marker genes such as the 18S ribosomal RNA (rRNA) gene (Lara et al., 2007a; Stoeck et al., 2006; Slapeta et al., 2005) suggest that ciliate diversity is much higher than proposed based on the described free-living morphospecies (Foissner et al., 2008). Such rRNA-based based molecular screening tools could complement or replace the direct observation and cultivation-dependent approaches and would moreover encourage also non-taxonomists to study this group of organisms. Especially fingerprinting techniques such as terminal restriction fragment length polymorphism (T-RFLP) (Euringer and Lueders,

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2008; Wu et al., 2009) or denaturing gradient gel electrophoresis (DGGE) (Diez et al., 2001; Wu et al., 2009) present appropriate tools for quick overall comparative analyses of protistan communities. However, only few surveys have extended the application of rRNA-based methods to soil systems (Lawley et al., 2004; Fell et al., 2006; Moon-van der Staay et al., 2006) and ecotoxicological aspects have not been addressed so far with these methods. Another major gap is the lack of primers specific for selected protistan taxa (Berglund et al., 2005; Rasmussen et al., 2001; Regensbogenova et al., 2004), particularly compared to that for bacteria. Protists are a polyphyletic group, thus making the design of specific rRNA gene targeting primers for various taxa necessary. Such primers should replace the commonly applied universal eukaryotic primers to allow for a more comprehensive picture of the protistan diversity and the protists' response to changing environmental parameters. The steadily increasing 18S rRNA gene databases harbour sequences from numerous cultured ciliates of all taxonomic subgroups (Dunthorn et al., 2008; Gao et al., 2008; Schmidt et al., 2007; Utz and Eizirik, 2007; Struder-Kypke et al., 2006) and from environmental clone libraries (Lara et al., 2007a; Behnke et al., 2006), which can consequently be used to develop rapid diagnostic tools.

Here we present a DGGE protocol for the rapid screening of ciliate communities in environmental samples. The method relies on a previously developed specific ciliate primer set which covers all major ciliate classes (Lara et al., 2007a). In order to detect also ciliates present in only low abundances, which might be typical for stressed environments, and to make the length of the amplicons compatible with the DGGE separation we included an additional semi-nested PCR step. The protocol was first evaluated on environmental clones covering a wide taxonomic range (Lara et al., 2007a). We then applied the method to environmental samples from a soil polluted with polycyclic aromatic hydrocarbons (PAH) and a close, unpolluted control soil with similar characteristics to assess the ability of the method to monitor pollution induced changes in ciliate communities.

2. Methods

2.1. Sample collection and DNA extraction

Soil samples were collected from a polycyclic aromatic hydrocarbon (PAH) contaminated site (total PAH content 2.8 g/kg dry soil) and a non-polluted control soil as described in Lara et al. (2007a). The contamination reflects 90 years of exposure history to creosote. Samples from the polluted soil were taken at 0, 10 and 20 cm depths. Additionally, control samples from the pristine soil were collected at 20 cm depth. Samples were frozen (−20 °C) until analysis. DNA was extracted using a bead beating method (Lara et al., 2007a).

2.2. PCR amplification

Ciliate 18S rRNA genes were amplified with a semi-nested PCR approach. The first amplification was carried out as described previously (Lara et al., 2007a) with the primers CilF and an equimolar mixture of three reverse primers CilR I, II and III (Table 1). Eight nanograms of the resulting product was reamplified using the forward primer CilF carrying a 36-bp GC clamp (Muyzer and Smalla, 1998) and the new reverse primer CilDGGE-r (Table 1), resulting in a ca. 600 bp product. PCR was carried out in a final volume of 50 µl containing 2 µl of DNA template, 1.25 U Taq Polymerase (Qiagen), 1× Reaction Buffer (Qiagen), 2 mM $MgCl_2$, 0.2 µM of each primer, 200 µM of each dNTP, and 3.6 mg ml^{-1} acetylated bovine serum albumin (BSA). The program set for the second amplification consisted of an initial denaturation at 94 °C for 5 min, followed by 26 cycles of 94 °C for 1 min, 52 °C for 1 min, and 72 °C for 1 min and a final elongation of 10 min at 72 °C to reduce double bands in the DGGE patterns (Janse et al., 2004).

Table 1
Primers used in this study.

Primer	Sequence 5′–3′	Position
CilF	TGG TAG TGT ATT GGA CWA CCA	315
CilF-GC	[GC clamp]TGG TAG TGT ATT GGA CWA CCA	315
CilR I	TCT GAT CGT CTT TGA TCC CTT A	959
CilR II	TCT RAT CGT CTT TGA TCC CCT A	959
CilR III	TCT GAT TGT CTT TGA TCC CCT A	959
CilDGGE-r	TGA AAA CAT CCT TGG CAA ATG	940

Positions are given in reference to *Tetrahymena australis* (X56167).

2.3. Denaturing gradient gel electrophoresis (DGGE)

Purified PCR products were quantified in a fluorometer (Turner design, Sunnyvale, CA, USA) using the PicoGreen stain (Molecular Probes, Eugene, USA). For each sample 250 ng of DNA was loaded on the gel. DGGE was performed in a D-Code system (Bio-Rad) with 0.75 mm-thick 6% polyacrylamide gels (acrylamide:bisacrylamide ratio 37.5:1) in 1× TAE buffer. Electrophoresis was carried out for 16 h at 58 °C and 80 V in a linear 32 to 42% denaturant gradient (100% denaturant defined as 7 M urea and 40% deionised formamide). Gels were stained with SYBR Green (Invitrogen) for 30 min, visualised with an UV transilluminator and analyzed with the software packages GeneSnap and GeneTools (SynGene, Cambridge, UK).

Two markers were designed to allow comparison of different gels: the first marker contained equal amounts (5 ng each) of amplified PCR products from 13 ciliate 18S rRNA gene clones covering a wide taxonomic range (Table 2). In order to detect possible preferential amplifications due to the semi-nested protocol, we additionally pooled equal amounts of PCR templates from the same clones used for the marker, reamplified this DNA mixture and loaded 65 ng of the resulting PCR product to the gel.

2.4. Sequencing of selected DGGE bands

Selected predominant bands were excised from the DGGE gels and transferred into a PCR tube containing 10 µl of MilliQ sterile water. Tubes were incubated overnight at room temperature to allow diffusion of the DNA, and 2 µl of the water was used as template for subsequent PCR reamplification. Cloned PCR products were subsequently sequenced with an ABI Prism® Big Dye™ terminator v 3.0 Ready Reaction Cycle Sequencing Kit (Applied Biosystems) on an ABI Prism® model 3100 automated sequencer. Sequences were aligned with the Clustal W algorithm (Thompson et al., 1994), adjusted manually and subjected to a BLAST search in GenBank to determine

Table 2
List of the clones used as marker.

Marker clone	GenBank accession number	Taxonomic affiliation
M1	DQ115964	Phyllopharyngea, Suctoria
M2	DQ115950	Oligohymenophorea, Hymenostomatida
M3	DQ115952	Oligohymenophorea, Scuticociliatea
M4	DQ115948	Oligohymenophorea, Peniculida
M5	DQ115959	Spirotrichea, Oligotrichea
M6	DQ115961	Spirotrichea, Hypotrichea
M7	DQ115943	Colpodea *incertae sedis*
M8	DQ115951	Oligohymenophorea, Scuticociliatea
M9	DQ115947	Nassophorea *incertae sedis*
M10	DQ115938	Colpodea, Colpodida
M11	DQ115936	Colpodea, Colpodida
M12	DQ115949	Oligohymenophorea, Peritrichea
M13	DQ115944	Phyllopharyngea, Cyrtophorida

All clones were obtained by Lara et al. (2007a).

the most closely related sequence in the database. Sequences were checked for chimeric structures with the CHIMERA_CHECK software program provided by the RDP (Cole et al., 2003) and controlled manually for conserved group-specific signature patterns.

2.5. Statistical analysis of DGGE fingerprints

DGGE fingerprints were converted to a presence–absence matrix, taking into account each band present in at least one sample as a single descriptor. The effect of PAH pollution on ciliate community structures was determined on the basis of their similarity matrix with a principal component analysis (PCA). Ciliate 18S rRNA gene richness as a measure of ciliate diversity was estimated by the total number of bands present in one sample. The effect of PAH contamination on the ciliate diversity was tested with a one way factorial ANOVA, followed by a Tukey HSD test ($\alpha = 5\%$). All statistical analyses were performed with R 2.4 (www.r-project.org).

3. Results and discussion

3.1. Evaluation of the semi-nested DGGE protocol

The optimization of the DGGE electrophoresis conditions using amplicons of 13 phylogenetically distinct ciliate 18S rRNA gene clone sequences (Table 2) resulted in a denaturing gradient of 32 to 42% and an electrophoresis time of 16 h. These conditions offered the highest resolution and separated most amplicons loaded in equal amounts into single sharp bands (Fig. 1). However, DGGE runs of an amplification product based on a mixture of the marker clones indicated a weaker amplification of the clones M1 (Oligohymenophorea, Hymenostomatida) and M2 (Phyllopharyngea, Suctoria) (Fig. 1.) Also, the spirotrichean clones M5 and M6, which show 3% sequence divergence, migrated to the same position in the gel and were not properly separated. An equal mobility of amplicons considerably differing in their sequence is a well-known bias associated with DGGE and shows that a single band does not necessarily imply a single sequence type (Sekiguchi et al., 2001). Thus, although DGGE patterns provide an overall quick community profile, richness estimation based on DGGE bands should only be used as an indication of the degree of diversity within communities.

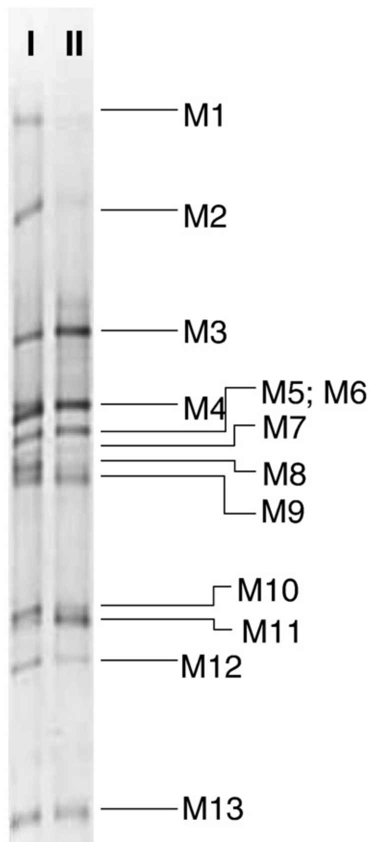

Fig. 1. DGGE profile of the 13 marker clones (M1–M13) used in this study. Left lane (I): separation of equal quantities of PCR-amplified single clone sequences. Right lane (II): separation after semi-nested PCR on a template consisting of pooled clones. See Table 2 for specific description of the mentioned clones.

The semi-nested PCR allows a more sensitive detection of target organisms that are present only in low abundances. Protist numbers in the unpolluted control soil (Lara et al., 2007b) were reported to be in general at the lower end of what is usually found in soils (Ekelund and Rønn, 1994), which we attribute to the low organic carbon content (0.04%) in the control soil. However, we cannot exclude that a second round of PCR amplification may increase differential amplification in complex template mixtures and thus influence the abundance of a sequence type in a DGGE gel (Suzuki and Giovannoni, 1996). Varying rRNA gene copy numbers may further blur the true reflection of sequence abundances in an environmental sample (Ward et al., 1997). The differential amplification of the clone M2 is also due to the mismatch in the CIL-DGGEr annealing site of clone M2, which is shared by many but not all Hymenostomatida. Using an additional degenerate primer may solve this problem but could also result in differently migrating PCR products from the same template. Another possibility is to further lower the annealing temperature in order to reduce preferential amplification as suggested by recent studies (Sipos et al., 2007; Ishii and Fukui, 2001). We therefore recommend, that further small primer optimisation might be necessary before using this DGGE protocol as a monitoring tool for samples with an expected high abundance of Hymenostomatida, such as freshwater environments.

3.2. Application of the DGGE protocol to soil ciliate communities

Sequence analysis of excised band confirmed that the semi-nested PCR protocol produced only amplicons from ciliates thus satisfying one fundamental condition required for environmental diagnostic screening tools, namely a high level of specificity. Given the fact that the amplified rRNA gene fragment included the most variable regions V3 and V4 (Wuyts et al., 2000), members from a wide range of ciliate groups could be successfully discriminated. Moreover, the comparatively large size of the amplified fragments allowed a reliable taxonomic affiliation of the species by sequencing of the bands.

DGGE profiling readily distinguished ciliate communities from PAH polluted and non-polluted sites and revealed between 5 and 15 bands per sample (Fig. 2). No variability was observed between ciliate DGGE patterns of replicate amplification from the same DNA extraction (data not shown). However, DGGE patterns within one site or within one depth appeared to be quite variable, confirming that ciliates are patchily distributed in soils (Acosta-Mercado and Lynn, 2002; Griffiths, 2002). This was in particular visible for the polluted soil samples which can be explained by the rather heterogeneous distribution of the creosote within the site due to its strong adsorption on clay particles and its low water solubility, thus potentially creating microhabitats with different pollutant loads.

The community fingerprints revealed that some bands were common to all samples, while others, especially in the lower part of the gel (at approx. 39% denaturant), appeared with increased intensity in particular in samples from polluted soils (Fig. 2). In general, PAH pollution had a very pronounced effect on community composition and number of phylotypes, i.e. richness. Richness in the polluted soil was reduced, resulting in an average richness of 5.7 bands as compared to 7.8 bands in the non-polluted control soil. As indicated by the principal component analysis (PCA) of the community profiles, DGGE allowed the clear

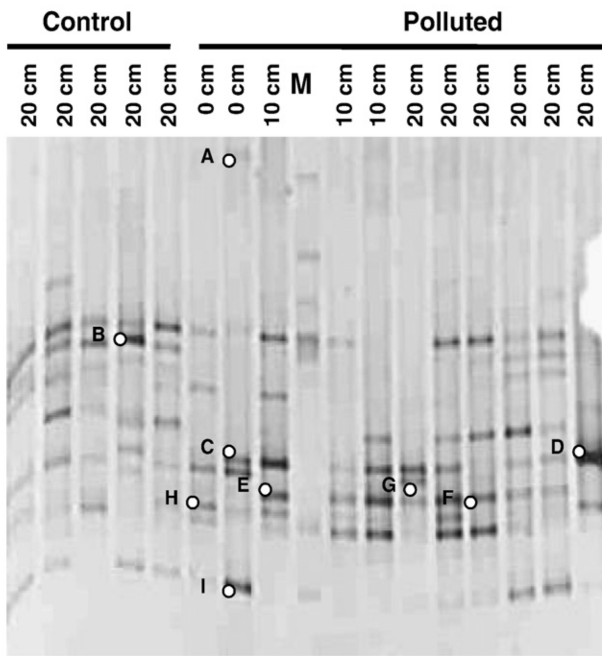

Fig. 2. DGGE profiles of ciliate communities in non-polluted control and polluted soil samples from different depths. Bands marked with a circle were sequenced (see Table 3).

Table 3
Phylogenetic affiliation and next BLAST hit of selected excised and sequenced DGGE bands.

Band	Best match	Sequence identity (%)	Taxonomic affiliation
A	Soil clone NPS05-2 (DQ115948)	97	Oligohymenophorea, Peniculia
B	*Arcuospathidium cultriforme* (DQ411860)	96	Litostomatea, Haptorida
	Soil clone NPS05-11 (DQ115955)	96	
C	Soil clone NPS05-139 (DQ115943)	98	Colpodea
D	*Colpoda steinii* (DQ388599)	98	Colpodea, Colpodida
	Soil clone NPS05-12 (DQ115937)	97	
E	Soil clone NPS05-27 (DQ1159389)	99	Colpodea, Colpodida
	Pseudoplatyophrya nana (AF060452)	96	
F	Soil clone NPS05-27 (DQ1159389)	100	Colpodea, Colpodida
	Pseudoplatyophrya nana (AF060452)	96	
G	*Colpoda cucullus* (EU039893)	97	Colpodea, Colpodida
	Soil clone NPS05-12 (DQ115937)	97	
H	*Colpoda steinii* (DQ388599)	97	Colpodea, Colpodida
	Soil clone NPS05-15 (DQ115936)	97	
I	*Arcuospathidium cultriforme* (DQ411860)	96	Litostomatea, Haptorida
	Soil clone NPS05-11 (DQ115955)	96	

separation of the ciliate communities from the polluted soil samples from those of the non-polluted control soil (Fig. 3). The first axis discriminated communities from non-polluted and polluted samples and explained 33.4% of the observed variance, suggesting that exposure to PAH was the major factor explaining the structure of the ciliate communities.

BLAST-searches of the excised sequences assigned them to several ciliate classes and showed that they were amongst others closely related (up to 100%) to those found by Lara et al. (2007a) in environmental clone libraries from the same soil (Table 3). We in particular focused on bands that migrated to the lower part of the gel (at approx. 39% denaturant), which turned out as the gel section characteristic for communities from the polluted soil. Interestingly most of these sequences were affiliated to members of the class Colpodea (Fig. 2; Table 3) and dominated the community patterns from the polluted soil samples. This is in general agreement with the number and abundance of dominant clone sequences obtained for the same soil by Lara et al. (2007a), indicating that the nested PCR approach with subsequent DGGE profiling obtained comparable results. Yet, DGGE detects mainly the predominant members of the community, while amplicons of substantially less abundant members might be hidden in the background smear. In addition, direct microscopic observation of enrichment cultures confirmed that Colpodea became the most abundant taxon in enrichments from the polluted soil (Lara et al., 2007a). Although we show some evidence that Colpodea might be characteristic for PAH polluted soils, further studies combining this DGGE approach with direct microscopic observation are required to prove this assumption.

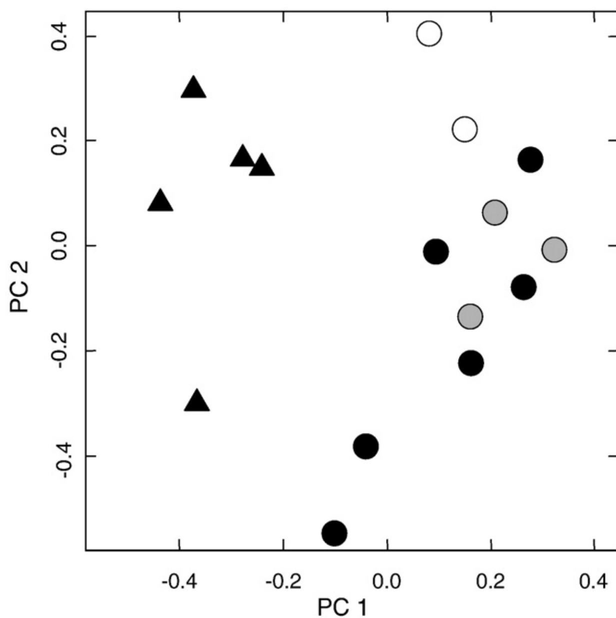

Fig. 3. Principal component analysis plot of DGGE profiles from the non-polluted control (triangle) and the polluted soil (circle) at a depth of 0 (open figures), 10 (grey) and 20 cm (black). The two first components explain 33.4% and 19.0% of the total variance, respectively.

In conclusion, we provide a target specific DGGE method as an alternative diagnostic tool to quickly monitor and compare overall ciliate community structures in polluted and unpolluted soils. We also envisage that the proven specificity of the first-round PCR primers enables the potential combination with novel group specific primers for the nested PCR step depending on the screening strategy and the ecosystem analyzed. Based on our results, it seems likewise advisable to carefully design an appropriate sampling strategy taking into account the spatial heterogeneity of soil ciliate communities before evaluating the bioindication potential of ciliate community structures and distinct populations.

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