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Molecular comparison of cultivable protozoa from a pristine and a polycyclic aromatic hydrocarbon polluted site

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Abstract

We compared the abundance and diversity of cultivable protozoa (flagellates and amoebae) in a polycyclic aromatic hydrocarbon (PAH) polluted soil and an unpolluted control, by isolating and cultivating clonal strains. The number of cultivable protozoa was higher in the polluted soil; however, the polluted soil displayed an impoverished community, dominated by certain taxa, such as *Acanthamoeba* sp. We isolated a total of 31 protozoan strains to characterize them morphologically and by 18S rRNA gene sequence analysis. This approach, i.e. combining morphological and molecular information had the advantage of providing quantitative data, information on morphology but also an accurate positioning of the isolates in 18S rRNA trees.

Keywords: 18S rRNA; Naked amoebae; Flagellates; Cultivable diversity; PAH polluted soil

1. Introduction

Decomposition and mineralization of natural and synthetic organic substances are crucial processes in terrestrial ecosystems, which strongly depend on interactions between various types of soil organisms (Moore and de Ruiter, 1991). Heterotrophic protozoa play a key role in the soil food web as they are direct consumers of primary decomposers such as bacteria and fungi (Foissner, 1987; Clarholm, 1994; Ekelund and Rønn, 1994; Ekelund, 1998). Protozoan grazing is an important key factor in shaping bacterial community composition in soils (Rønn et al., 2002), freshwater ecosystems (Jürgens et al., 1999) and activated sludge (Güde, 1979). Bacterial properties such as size, shape and cell wall composition play a role in protozoan selection of specific bacterial prey (Singh, 1942; Bianchi, 1989; Shikano et al., 1990; González et al., 1990; Gurijala and Alexander, 1990; Jezbera et al., 2005).

Protozoan grazing pressure also influences physiological properties and activities of bacterial populations by stimulating a number of bacterial activities, e.g. CO_2 evolution (Elliot et al., 1980), methanogenesis (Biagini et al., 1998), nitrification (Griffiths, 1989) and nitrogen fixation (Hervey and Greaves, 1941). There is also evidence that bacterivorous protozoa may influence pollutant degradation processes (Kinner et al., 2002). Toluene consumption by a *Pseudomonas* strain in batch culture was up to 7.5 times faster when the soil flagellate *Heteromita globosa* was present (Mattison and Harayama, 2001), and grazing by the ciliate *Colpidium colpoda* enhanced the degradation of crude petroleum in batch culture (Rogerson and Berger, 1983). Still, the abundance and diversity of protozoan communities in hydrocarbon-contaminated environments are poorly documented; Rogerson and Berger (1981), for example, found that crude oil pollution neither affected seriously neither trophic nor encysted protozoa. Naked amoebae were observed in oil-impacted salt marshes (Anderson et al., 2001), whereas seven genera of flagellates and five genera of naked

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amoebae were identified in cultures obtained from a sewage-contaminated aquifer (Novarino et al., 1994). Coupe et al. (2003) examined the protozoan diversity in cultures containing some of the different chemical constituents used in pavement construction and mineral oil. They observed mainly flagellates but also naked amoebae, ciliates and testate amoebae. However, none of these studies included molecular data and in Anderson et al. (2001) the morphological description was even restricted to morphotypes.

Studies of the microbial diversity that rely exclusively on retrieval of DNA sequences are limited by some serious pitfalls. For example, so-called "standard universal primers" are often not universal, and fail to amplify some prokaryotes (Von Witzingerode et al., 1997) and eukaryotes (Berney et al., 2004). Consequently, several taxa are very likely overlooked. In addition, selective DNA extraction, polymerase chain reaction (PCR) and cloning procedures further bias the picture of the community. In eukaryotes, for examples, during DNA extraction, the cells of certain, often amoeboid, taxa are less easily disrupted (Berney et al., 2004). Moreover, environmental surveys that entirely rely on cloning and sequencing hardly give reliable information on the number of organisms present (Von Witzingerode et al., 1997).

Here we report on the results of an alternative approach, where we used traditional methods to quantify and isolate flagellates and naked amoebae, which we then characterized by sequencing their 18S rRNA genes. We applied this approach to polycyclic aromatic hydrocarbon (PAH) polluted and non-polluted samples from the same soil, a mineral soil with a very low organic matter content. First, abundances of protists in the soil using an most probable number (MPN) approach were estimated, and monoxenic protozoan cultures from the soil samples were established and identified using light microscopy. Subsequently, their 18S rRNA genes were sequenced to infer their positions in the eukaryotic 18S rRNA tree.

2. Materials and methods

2.1. Soil samples

Soil was sampled from a PAH-contaminated spot at a former railway station in Andújar (Province of Jaén, Spain) and from an uncontaminated area located nearby. The PAH contamination is a result of the treatment of railway sleepers with creosote during the last 90 years. The contaminated soil was a silty clay (3.4% sand, 37% silt and 59.5% clay), and had a total PAH content of $2.7\,g\,kg^{-1}$ dry soil. The control site located just outside the contaminated area (3 m away) was likewise a silty clay (1.4% sand, 31.1% silt, 67.4% clay) having an organic matter content of only $0.4\,g\,kg^{-1}$ and containing only traces of PAH. Eight samples where taken from the polluted soil, and five from the unpolluted control. All samples were kept in the dark at 15 °C until further analyses; they were kept under these conditions for 6 months prior to serial dilutions and MPN counting. This high storage temperature was chosen in order to keep the soil microflora under realistic conditions for the region of origin of the samples.

2.2. MPN counting

We used a modified MPN approach (Rønn et al., 1995) to estimate the numbers of naked amoebae and heterotrophic flagellates in the soil samples. The method is based on a threefold dilution series culturing technique using 96-well microtitre plates (Sarstedt, Newton, USA). We used $0.1\,g\,l^{-1}$ tryptic soy broth (Difco, Detroit, USA) as culture medium (Rønn et al., 1995). The microtitre plates were placed in the dark at 13 °C, and protist growth was verified in single wells by examination with an inverted microscope 1 and 3 weeks after starting the serial dilutions. The number of flagellates and the number of naked amoebae were estimated for each soil sub-sample, and average values and standard deviation were calculated for each soil type.

2.3. Isolation and cultivation

We attempted to isolate as many pure cultures as possible from the microtitre plates used for protozoan enumeration. The contents of the wells that seemingly contained only one protist morphotype were sequentially diluted again in a 96-well microtitre plate, and after repeated growth the resulting cultures were transferred to 50 ml Nunc-flasks containing Neff's Amoeba saline medium (Page 1988) with $0.1\,g\,l^{-1}$ tryptic soy broth, and kept at 11 °C. Careful examination by light microscopy ensured the absence of contaminating protozoa, yeasts or fungal hyphae. Microscopic observations were compared with several manuals and publications displaying identified protozoa (Page, 1988; Ekelund and Patterson, 1997; Tong et al., 1998; Atkins et al., 2000; Lee and Patterson, 2000). Approximately every 2 months, cultures were transferred to fresh medium.

2.4. DNA isolation, PCR and sequencing

Cultures designated for DNA extraction were grown as dense as possible. The bottom of the culture flask was scratched with a sterilized pipette tip to detach surface-associated individuals and cysts. For extraction, 380 μl of culture medium was mixed with 20 μl lysis buffer (10% SDS, 0.1 M NaCl, 0.5 M Tris, pH 8.0) and 40 μg proteinase K, incubated at 45 °C for 2 h, and subsequently extracted with phenol:chloroform:isoamylalcohol (25:24:1). The DNA was precipitated with ethanol at −20 °C overnight. After centrifugation (12,000g for 10 min at 4 °C) the DNA-pellet was washed in 70% ethanol, air-dried and resuspended in 25 μl of sterile double-distilled water.

PCR was performed with a PTC-200 thermal cycler (MJ Research, Waltham, USA) using a combination of the

universal 18S rRNA gene forward primer 5′-GACGGG CGGTGTGTACA-3′ and the reverse primers 5′-CTGGTT GATCCTGCCAG-3′ or 5′-TGATCCTTCYGCAGGTT-CAC-3′, respectively (Sogin and Gunderson, 1987; Moon-Van der Staay et al., 2001). The following conditions were applied: 30 cycles (denaturation at 94 °C for 45 s, annealing at 55 °C for 30 s, extension at 72 °C for 2 min) followed by a 5-min denaturation at 95 °C and followed by a 10-min extension at 72 °C. PCR products were checked on a 1% agarose gel and purified using the Nucleospin Extract kit (Macherey Nagel, Düren, Germany). PCR products were either sequenced directly or inserted in a pGEM-T Easy cloning vector and used to transform *Escherichia coli* JM109 competent cells (Promega, Madison, WI, USA).

Sequencing was carried out with an ABI Prism Big Dye^{TM} Terminator v 3.0 Ready Reaction Cycle Sequencing Kit (Applied Biosciences) and an ABI PRISM 3100 automated sequencer, using the conserved eukaryotic primers nu-SSU-1288, nu-SSU-598 (ordered both as forward and reverse sense primers) and nu-SSU-898 in the forward sense (Willerslev et al., 1999).

2.5. Database search and phylogenetic analysis

BLAST searches (Altschul et al., 1990) were performed with the new 18S rRNA gene sequences to obtain closely related or otherwise phylogenetically relevant sequences. Five different alignments were made, one for each of the following groups: Chrysophyceae, Kinetoplastida, Amoebozoa, Jakobida-Heterolobosea, (this alignment comprises also the jakobid isolates), and Cercomonadida. The 18S rRNA gene sequences were aligned manually using the Genetic Data Environment software, version 2.2 (Larsen et al., 1993), following the secondary structure model proposed by Wuyts et al. (2000). Additional sequences from public databases were selected to optimally illustrate the phylogenetic position of the isolated organisms. All phylogenetic analyses were performed using the GTR model of substitution (Lanave et al., 1984; Rodriguez et al., 1990), taking into account a proportion of invariable sites, and a gamma-shaped distribution of the rates of substitution among variable sites, with eight rate categories. The number of unambiguously aligned positions that were used in our phylogenetic analyses were, respectively, 1531, 1673, 1397, 1330, and 1363, for the Chrysophyceae, the Kinetoplastida, the Amoebozoa, the Jakobida-Heterolobosea, and the Cercomonadida datasets (Proportion of invariable sites: 52.3%, 38.7%, 12.6%, 20.4%, 40.2%, respectively; Alpha parameter: 0.2986, 0.3825, 0.4271, 0.7884, 0.3983, respectively). All necessary parameters were estimated from the datasets. For each dataset, an evolutionary tree was inferred with the maximum likelihood method (Felsenstein, 1981) using the program PhyML, version 2.0.3 (Guindon and Gascuel, 2003). The reliability of internal branches was assessed with the posterior probabilities calculated by Bayesian analyses, which were performed with MrBayes, version 3.0b4 (Huelsenbeck and 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. Alternatively, the bootstrap method (Felsenstein, 1985) was used for minimum evolution analyses, with 10,000 replicates. Minimum evolution analyses were performed with maximum likelihood-corrected estimates of the distances, using the BioNJ option in PAUP*, version 4.0b10 (Swofford, 1998). The sequences reported in this paper have been deposited in the GenBank database under the accession numbers from AY965859 to AY965873, plus AY994316 and called And1-32.

3. Results

3.1. MPN counts

Naked amoebae dominated the protozoan community in the polluted soil with MPN counts of $2.5 \times 10^4\,g^{-1}$ dry soil, i.e. significantly more ($P = 0.012$, one way ANOVA on log-transformed data) than the 7×10^2 amoebae g^{-1} dry soil in the unpolluted soil. In contrast we only recorded 1.3×10^3 flagellates g^{-1} dry soil in the polluted soil, whereas we recorded a significantly ($P = 0.014$, one way ANOVA on log-transformed data) higher number, $6.5 \times 10^3\,g^{-1}$ dry soil, in the unpolluted soil (Fig. 1).

3.2. Morphological examination

We isolated and cultured 31 different strains of heterotrophic protozoa from the soil samples. Three naked amoebae and 12 heterotrophic flagellates were isolated from the pristine soil; 10 naked amoebae and six heterotrophic flagellates originated from the polluted soil samples. We grouped the strains to the lowest possible taxonomic level by morphological criteria using light microscopy, but for most of the strains, e.g. the strains with a cercomonas-morphology this was not satisfactory.

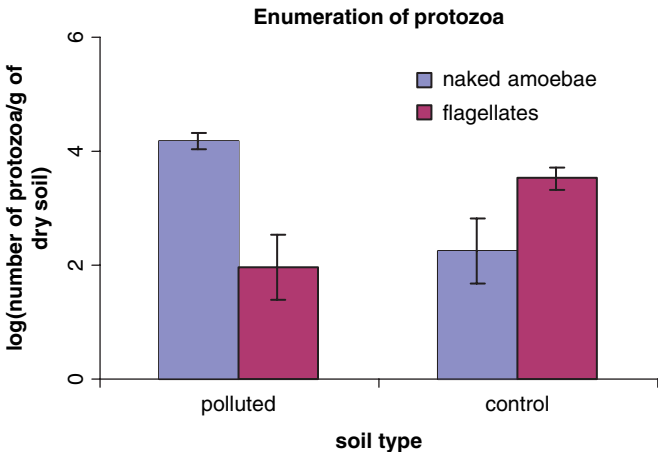

Fig. 1. Plot representing the logarithms of the numbers of naked amoebae and flagellates in pristine and polluted soil as calculated by MPN ($\log(\text{mean} \times \text{g soil}^{-1}) \pm$ standard error).

Table 1
List of the clonal isolates discussed in the paper, with morphological notes and taxonomic affiliation

Isolate	Isolated from	Taxonomic affiliation	Size (μm)	Pseudopodia	Flagella	Locomotion	Special comments
And11	Control soil	Rhizaria, Cercomonadida	5	absent	Single trailing flagellum	Gliding, surface-associated	
And3	Control soil	Rhizaria, Cercomonadida	5–11	Thin and sometimes branched, only from posterior half of the cell	Two flagella, posterior 2 times cell length	Gliding, surface-associated	
And18	Control soil	Rhizaria, Cercomonadida	5–10	Filamentous, formed at any part of the cell	Two flagella, posterior 1–1.5 times cell length	Gliding, surface-associated	Refractile granule present, visible also in cysts
And13, And24	Control soil	Rhizaria, Cercomonadida	5	Sometimes with a pseudopodial tail	Two heterodynamic flagella, both about 1 time cell length	Wobbling, surface-associated	
And30	Control soil	Chromalveolata, Chrysophyceae	4–5	Absent	Two flagella, anterior about 2 time cell length, posterior 0.5 time	Free-swimming, sometimes attached, arched moving pattern	Large vacuole
And15, And25	Control soil	Rhizaria, Cercomonadida	10–16	Sometimes in the posterior part	Two flagella, anterior about 2 time cell length, posterior 1 time	Gliding, surface-associated	
And27	Control soil	Rhizaria, Cercomonadida	6–10	Sometimes in the posterior part	Two flagella, anterior about 1.5 times cell length	Gliding, surface-associated	
And16	Control soil	Amoebozoa incertae sedis	20–30	Long, branched and filamentous	Absent	Surface-associated	Cysts with a small excystment "plug"
And19	Control soil	Excavata, Jakobida	3–5	Absent	Two flagella of equal size, the trailing flagellum beating in a ventral groove	Free-swimming, rotating movement	Morphologically similar to And28
And12	Control soil	Excavata, Heterolobosea	20–30	Lobose, with hyaline margin	Absent	Surface-associated	Short bulbous uroid, cysts without any visible pores
And9	Control soil	Excavata, Heterolobosea	20	Lobose, with hyaline margin	Absent	Surface-associated	Monopodial
And31	Control soil	Excavata, Euglenozoa, Kinetoplastida	10–12	Absent	Two flagella inserted subapically, posterior flagellum 3 times cell length	Partially free-swimming, sinuous moving patterns	Refractile granules at the end of the cell
And21, And1	Control and polluted soil	Rhizaria, Cercomonadida	5	Absent	Two heterodynamic flagella, both about 1 time cell length	Wobbling, surface-associated	
And6	Polluted soil	Rhizaria, Cercomonadida	5–10	Sometimes in the posterior part	Two heterodynamicflagella, anterior about 1 time cell length	Gliding, surface-associated	Granules inside the cytoplasm, dark and refractile
And28	Polluted soil	Excavata, Jakobida	3–5	Absent	Two flagella of equal size, the trailing flagellum beating in a ventral groove	Free-swimming, rotating movement	Ventral groove in the cell body
And32-41	Polluted soil	Amoebozoa, Acathamoebidae	20–25	Short and filamentous	Absent	Surface-associated	Cysts with thin intine, triangular shaped
And5, And10, And29	Polluted soil	Chromalveolata, Chrysophyceae	3	Absent	Two flagella, anterior about 2 time cell length, posterior 0.5 time	Free-swimming, sometimes attached, straight line moving pattern	

Hence, in addition we examined and compared their 18S rRNA gene sequences in order to assign them to a taxonomic group (Table 1). All the 31 isolated strains were sequenced. The cultivated protozoan communities in contaminated and control soils were quite different; only one strain, a cercozoan flagellate, was found in both soil types. The classification of the isolates has been indicated following the nomenclature of Adl et al. (2005). Some morphological characters of the isolates are given in Table 1.

3.3. Phylogenetic position of the isolates

The phylogenetic analysis in general confirmed the morphological identification. However, the phylogenetic analysis provided additional and more precise information on the identity of some of the isolated organisms (Figs. 2–6). For instance, And16 was placed within a group composed by environmental sequences and also the amoebae *Gephyramoeba* sp. and *Filamoeba nolandi*. The naked amoeba And12 was placed near the slime mould *Acrasis rosea*, and the heterolobosean amoeba And9 with the marine amoeba *Neovalkampfia damariscottae*. And3 and And18 were placed inside the genus *Cercomonas,*

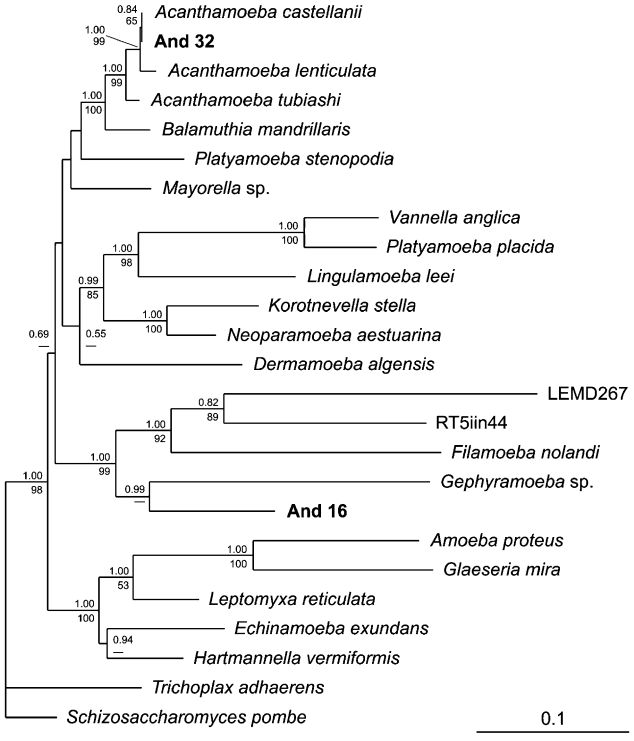

Fig. 2. Maximum-likelihood tree of lobose amoebae, including isolates And32 and And16. The tree was rooted with two opisthokonts. The numbers above the branches on each node indicate the posterior probabilities as calculated with Bayesian analyses, the numbers below indicate the levels of bootstrap support (percentages of 10,000 replicates) obtained in a minimum evolution analysis. Values under 0.5/50% are omitted. All branches are drawn to scale.

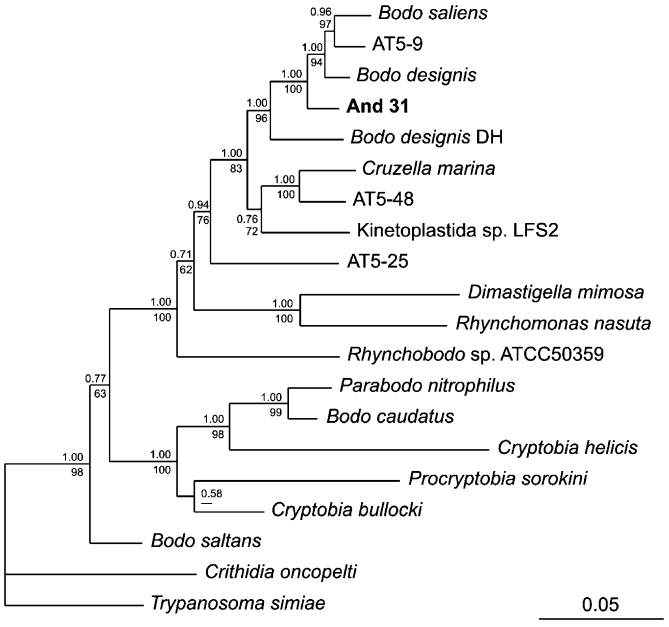

Fig. 3. Phylogeny of bodonids, including isolate And31. The tree was rooted with two trypanosomatids.

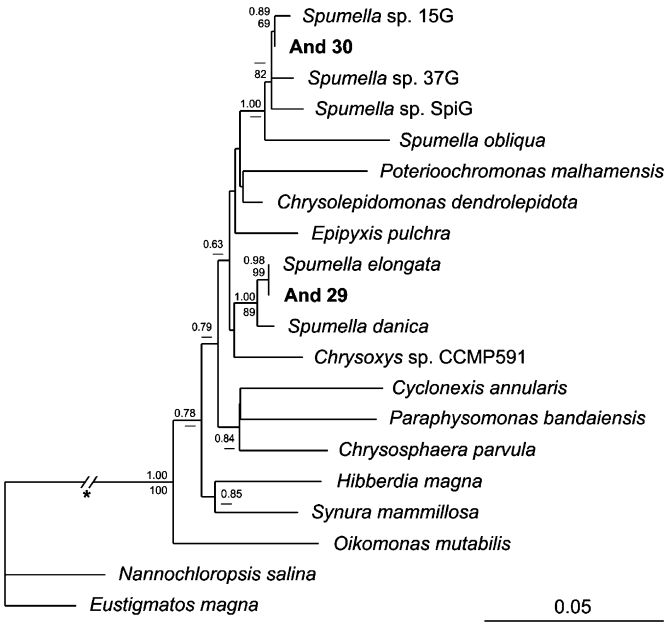

Fig. 4. Phylogeny of chrysophytes, including isolates And29 and And30. The tree was rooted with two eustigmatophytes. The branches marked with an asterisk are reduced from half for clarity.

whereas And6, 15 and 25 belonged to genus *Neocercomonas*. To date, no single criteria have been identified to separate these two genera morphologically.

4. Discussion

4.1. The experimental approach

We retrieved 31 different strains of heterotrophic protozoa from the two soils. We are naturally aware that

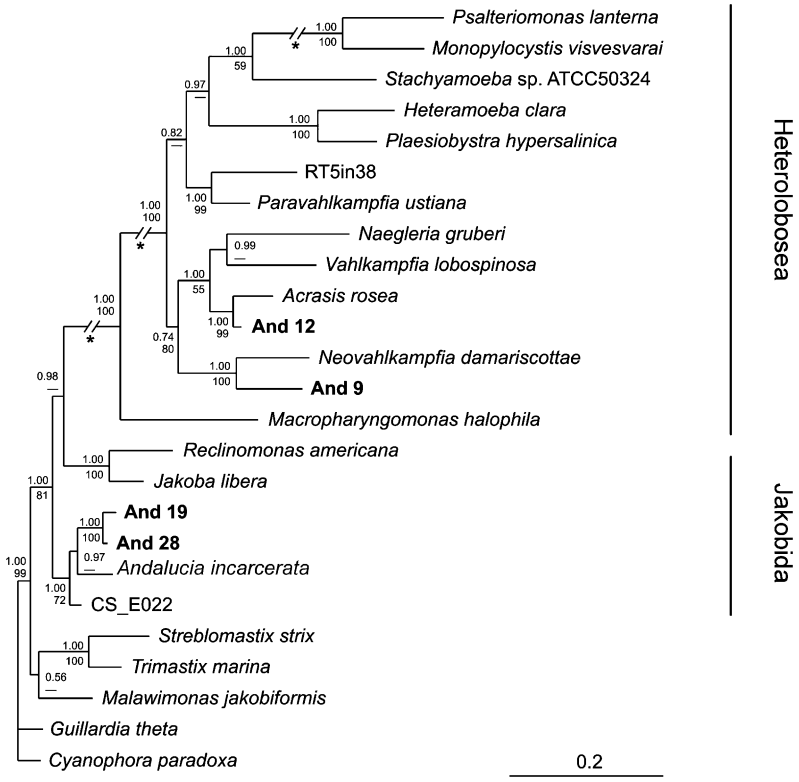

Fig. 5. Phylogeny of selected excavates, including isolates And19 and And28 (jakobids) and isolates And9 and And12 (heterolobosea). The tree was rooted with *Cyanophora paradoxa* and *Guillardia theta*. The branches marked with an asterisk are reduced from half for clarity.

the total soil protozoan diversity is larger. Culture-independent molecular surveys have revealed a large diversity of protozoa in various ecosystems (López-García et al., 2001; Moon-Van der Staay et al., 2001). Cultivation-dependent approaches may be hampered by different factors. Some protozoa do not survive the technical procedures associated with dilution techniques (Ekelund and Patterson, 1997), and some appear to be unable to grow on the bacterial food source offered in the microtiter plates (Severtsova, 1928; Mitchell et al., 1988; Boenigk and Arndt, 2002), or need an agar substrate for optimal growth. In addition, the condition of an homogeneous mixing of the soil suspension is hardly met (Berthold and Palzenberger, 1995). However, molecular analyses are known to be biased as well, and the approach we used allowed us to identify many forms that tend to be underrepresented in environmental DNA surveys, such as, for instance, amoeboid forms. For example, sequences of the ubiquitous Acanthamoebidae have, to our knowledge, never been found in published clone libraries of 18S rRNA gene sequences. This has been ascribed to reported difficulties to extract and amplify DNA from these organisms as has been observed even with pure cultures as the starting material (Berney et al., 2004).

The present approach combined light microscopy and DNA sequence information, and provided therefore additional information about the correlation between species presence and habitat.

For instance, the presence of the diagnostic sequences for *Heteromita globosa* as suggested by Ekelund et al. (2004) indicated that the isolates And21 and And1 belonged to that species. However, the partial sequence of the environmental clone LEMD045 (Dawson and Pace, 2002) had 99% identity with sequence And21, although LEMD045 had been retrieved from anoxic sediment. To our knowledge, all *H. globosa* strains reported until now were isolated from aerobic environment. Hence we conclude that either the sequence found by Dawson and Pace (2002) came from a cyst that survived the anoxia, that there are facultative anaerobic strains of *H. globosa* or that 18S rRNA sequences do not provide sufficient resolution to distinguish closely related, but ecophysiologically distinct protozoa (Boenigk et al., 2005).

4.2. The impact of polycyclic aromatic hydrocarbons on the community

Quantitative information about the abundance of the distinct types of protozoa was used to assess the effect of hydrocarbon pollution on the communities. The detected number of protozoa in the uncontaminated soil was 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 soil. In the polluted soil, the protozoan abundance was significantly higher, and in accordance with previous studies dealing with protozoan

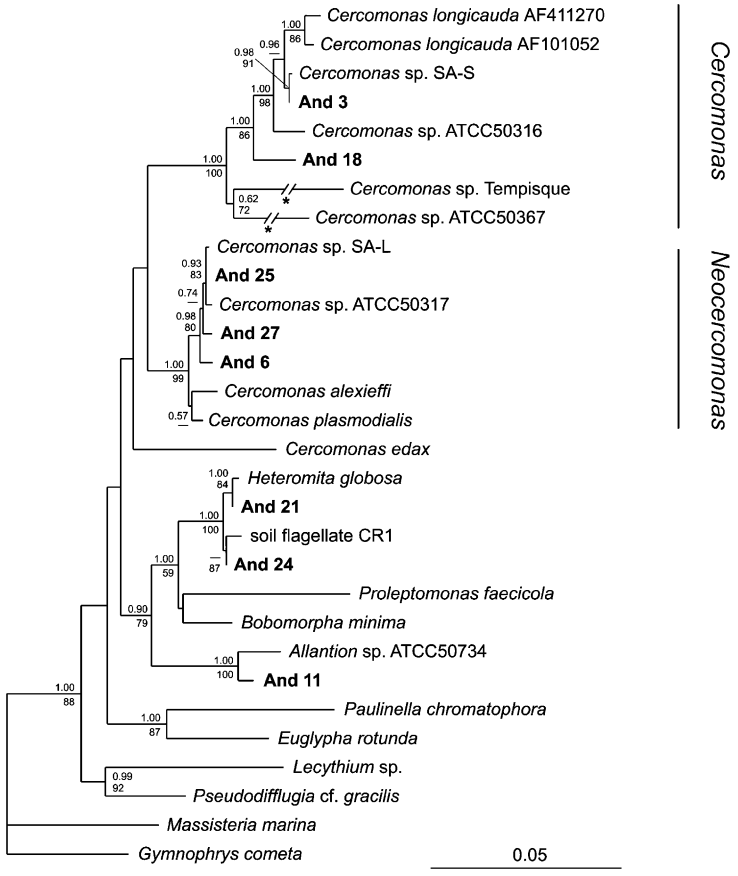

Fig. 6. Phylogeny of cercomonads including isolates And3, And6, And 11, And18, And21, And24, And25, and And27. The tree was rooted with *Massisteria marina* and *Gymnophrys cometa*. The branches marked with an asterisk are reduced from half for clarity.

abundance in organically polluted subsurface sites (Madsen et al., 1991; Sinclair et al., 1993). The reason for this is probably that the additional carbon substrates facilitate an increased bacterial growth. In our case, the PAH contamination increased the organic matter content by a factor of 10, thereby providing a significant additional amount of low-molecular weight carbon substrates to the microbial community.

However, the proportion of different strains of protozoa was lower in the polluted soil (30%) than in the control soil (85%), see Table1, suggesting a higher protozoan diversity in the unpolluted soil. The contamination with PAH probably affects protozoan diversity directly via its toxicity and indirectly via its effect on the bacterial prey (Ekelund, 1999). Toxic effects of PAH in the soil we studied have already been observed on the soil microfauna (Gillet and Ponge, 2005), and, generally toxicity of PAH compounds to soil organisms, other than protozoa, is well documented (Edwards and Fletcher, 1971; Dorn et al., 1998; Crouau et al., 1999).

Heterotrophic bacterivorous flagellates related to the cercomonads dominated the cultivable protozoans in the control soil. We isolated forms affiliated to the genera *Cercomonas*, *Neobodo*, *Neocercomonas*, *Heteromita* and *Allantion*. *Cercomonas* and *Heteromita* are common in soil environments, often making up more than 50% of the total protozoan biomass (Sandon, 1927; Foissner, 1991; Ekelund and Patterson, 1997; Ekelund et al., 2001), which is in accordance with our results. Our finding of two strains related to jakobids in soil was more unexpected since these flagellates have previously been reported only from marine sites (Vørs et al., 1994; Bernard et al., 2000).

Naked amoebae were relatively more abundant in the polluted soil, where they made up the largest part of the protozoan biomass. In the polluted soil, all cultivable amoebae had a morphology corresponding to *Acanthamoeba*. All 10 isolates obtained had an identical 18S rDNA gene sequence, suggesting a close relationship to *A. castellani*. The apparent lack of *Acanthamoeba* in the unpolluted soil, though, is rather surprising, since *Acanthamoeba* species are considered very common in soils (Page, 1988; Rodríguez-Zaragoza and García, 1997; Robinson et al., 2002). It could be explained, however, by the large food requirements of this amoeba (Bryant et al., 1982), which are probably not met in the unpolluted, mineral soil. In the control soil, other forms of heterolobosean (And9 and And12) and amoebozoan (And16) amoebae were found.

4.3. The phylogenetic position of the isolates

The two isolates related to jakobids, And 19 and And 28, proved to be the most basal branching organisms within

this clade. And28 has been now formally described as a new species, *Andalucia godoyi*, on the basis of molecular phylogeny and ultrastructure. This isolate was also the basis for the erection of a new genus comprising the new species plus the former *Jakoba incarcerata*, now *Andalucia incarcerata* (Lara et al., 2006).

And16 also nicely illustrates how genetic data can lead to unexpected and interesting conclusions about the identity of environmental isolates. Firstly, And16 clustered with a clade that only contains the morphologically and genetically very different *Filamoeba* and *Gephyramoeba*, as well as two environmental sequences (Amaral Zettler et al., 2002). Secondly, there is some evidence that the plasmodial slime moulds could originate from this clade (Fahrni et al., 2003). This sequence, together with other sequences from environmental isolates that may still await discovery, could help to understand how the relatives of *Physarum* evolved from solitary amoebae. Likewise, the vahlkampfid amoeba And12 clusters with the cellular slime-mould *Acrasis rosea*, with a high bootstrap value (99%). We could not expect to observe any aggregation formation, in our liquid cultures; however, it cannot be excluded that aggregation formation could take place during cultivation on agar. It would be interesting to isolate other free-living vahlkampfiid amoebae closely related to this group, in order to reconstruct a robust phylogeny of this clade that would provide a framework to study the evolution of structural traits such as aggregation formation.

5. Conclusion

The number of sequences from unidentified organisms provided by culture independent environmental surveys has grown rapidly during the last years. These new sequences from unidentified organisms doubtlessly increase our understanding of the evolution and diversity of unicellular eukaryotes. However, sequencing alone does not provide information about the ecological role of the organisms. Conversely, ecological investigation that does not take into account genetic diversity eludes the possibility to detect different ecophysiology of morphologically similar isolates. By employing a cultivation-dependant method combined with sequencing of the 18S rRNA gene of the isolates, we are likely to obtain a broader knowledge of the taxa present in soil as well as quantitative data.

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