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A molecular approach to the characterization of the eukaryotic communities of an extreme acidic environment: Methods for DNA extraction and denaturing gradient gel electrophoresis analysis

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Abstract

The diversity of the phytobenthonic community present in six acidophilic microbial mats from Río Tinto (Iberian Pyritic Belt, SW Spain) was analysed by optical microscopy and two molecular techniques, denaturing gradient gel electrophoresis (DGGE) and sequence analysis of 18S rDNA cloned gene fragments. Sixteen DNA isolation protocols as well as two commercial DNA extraction kits were tested and their efficiency compared. Purified DNA extracts were amplified by PCR using universal eukaryotic primers and the PCR products analysed by DGGE. Bead-mill homogenization was found to be superior to the other cell lysis methodologies assayed (sonication or freeze-thawing cycles) as it allowed efficiencies of cell disruption of over 95%. The methods combining bead-mill homogenization in the presence of SDS, treatment with chemical extractants (hexadecylmethylammonium bromide or guanidine isothiocyanate) and phenol extraction resulted in DNA preparations that amplified the same number of bands when analysed by DGGE as the two commercial kits assayed. The phylogenetic affiliations of the DGGE bands were determined by a BLAST search, and nine different species related to the *Chlorophyta*, *Ciliophora*, *Kinetoplastida*, *Ascomycota*, *Streptophyta* and *Colcochaetales* taxonomical groups were identified. Similar levels of diversity were found using cloning procedures. Although not all the species observed under the microscope were detected using molecular techniques, e.g. euglenas, heliozoan, or amoebae, DGGE fingerprints showed rather well the level of diversity present in the samples analysed, with limitations similar to cloning techniques.

Keywords: Ecology; Extremophiles; Microbial diversity; Acidophilic eukaryotes; Denaturing gradient gel electrophoresis (DGGE); DNA extraction

Introduction

Although the identification and quantification of organisms is routinely performed in microbial ecology, they still have limitations that only careful comparative analysis make evident [3,23]. Small phototrophic and heterotrophic eukaryotes are frequently found in many ecosystems and their identification and classification, usually based on morphological characteristics, is difficult due their size, similarity and lack of distinctive taxonomic properties. In the same manner, traditional cultivation techniques for enrichment and isolation of

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different species yield only a limited fraction of all microorganisms present.

In order to identify a larger fraction of species in natural samples, several molecular techniques have been developed over the last 15 years [18,26,33]. PCR-based molecular methods offer a fast and sensitive alternative to conventional cultivation techniques, because nucleic acids can be extracted directly from environmental samples, amplified and made amenable to nucleotide sequence comparative analyses [28,39]. The use of this approach shows us that microbial diversity is much greater than previously thought, and that isolation and culture techniques are insufficient to carry out these studies accurately.

Some techniques in molecular ecology are based on genetic fingerprinting which provides a pattern of the different species present in the community on the basis of the physical separation of their nucleic acid sequences [30]. In this respect, separation of DNA fragments by denaturing gradient gel electrophoresis (DGGE), has been used in an increasing number of microbial ecology studies [20].

Although DGGE has been very successful for describing bacterial diversity in a large number of ecosystems [9,19,22,27], only a few studies have used this technique to characterize the diversity of eukaryotic organisms. Most of the analysis of genes coding for 18S rRNA were carried out in fungal communities [34,36] and, only recently have some studies of marine picoeukaryotes [5], sludge bioreactors [16] and freshwater lagoons [37] focused on the whole eukaryotic assemblage revealing in all cases an unexpected level of diversity.

In the same regard, interest in the study of biodiversity in extreme environments has grown over the past years for several reasons, mainly biotechnological. One group of extremophiles that is becoming increasingly important, both ecologically and economically, corresponds to acidophilic organisms that live in low pH environments (pH < 3). Río Tinto (Iberian Pyritic Belt, Southwestern Spain) corresponds to a 92 km long river with a constant acidic pH (mean value 2.3) and high concentrations of heavy metals [14,15]. In spite of its physicochemical characteristics, eukaryotic organisms are the principal contributors to the biomass in this habitat, showing an unexpected degree of diversity [1,15].

In this study, we examined the suitability of the DGGE technique for the study of the eukaryotic diversity present in extreme acidic environments. The relative effectiveness of different DNA extraction protocols was tested with two environmental samples from Río Tinto. Finally, the DGGE results were compared with those obtained using gene cloning and microscopy observations.

Materials and methods

Sampling and microscopic species identification

Six different samples were taken from Río Tinto by carefully scraping biolayers from the upper part of sediments with sterile razor blades. Samples were transported to the laboratory in sterile 25 ml plastic tubes, centrifuged (10 min at 8000*g*), the supernatant was removed and cells were frozen and stored at −20 °C until DNA extraction.

Identification of algae and heterotrophic protists was carried out by direct microscopic observation using different phenotypic features based on previous studies of the eukaryotic communities in this river [1,15]. A Zeiss Axioscope 2 microscope equipped with phase-contrast was used for optical microscopy. Cell counts were performed in triplicate in a Sedwerick–Rafter chamber.

DNA isolation

Sixteen DNA extraction methods differing in cell lysis treatment were compared (Table 1). These methods can be classified as (i) mechanical lysis, (ii) mechanical lysis followed by phenol extraction, (iii) mechanical lysis in the presence of SDS, followed by chemical treatment, and a final phenol extraction, (iv) mechanical lysis in the presence of SDS, followed by chemical treatment, enzymatic incubation and phenol extraction and (v) commercial kit extractions. Each sample was divided into two 1 ml subsamples and DNA was extracted independently from duplicate sets.

(i) Mechanical lysis: After washing the samples five times with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8), three different mechanical treatments were used:
- Method 1: sonication. Cells resuspended in 0.5 ml TE buffer were sonicated for 5 min using pulses of 1 min on ice in a Mixonic XL sonicator with an output frequency of 20 kHz.
- Method 2: freeze-thaw cycles. Samples were frozen for 2 min in liquid N_2 and immediately placed in a boiling water bath for 1 min to rapidly thaw the sample. This process was repeated 5 times.
- Method 3: Bead-mill homogenization. Samples were homogenized for 40 s at 5500 rpm in a bead beater cell disruptor (FastPrep Instrument, Bio 101, Carlsbad, CA, USA). Samples were placed in 2 ml bead beater tubes containing 1.8 g of a mixture of ceramic beads (1/4″ spheres and a garnet matrix). This bead combination is able to disrupt soil colloids, plant tissues, fungal and other eukaryotic cells as well as bacterial cells.

Table 1. Cell lysis and DNA extraction methods

Method	Cell lysis treatment		
	Mechanical	Chemical	Enzymatic
1	Sonication		
2	Freezing–thawing		
3	Bead beating		
4	Sonication	Phenolization	
5	Freezing–thawing	Phenolization	
6	Bead beating	Phenolization	
7	Sonication	SDS 1%-CTAB	
8	Bead beating	SDS 1%-CTAB	
9	Sonication	SDS 1%-GIT	
10	Bead beating	SDS 1%-GIT	
11	Sonication	SDS 1%-CTAB	Pronasa/lysozyme
12	Bead beating	SDS 1%-CTAB	Pronasa/lysozyme
13	Sonication	SDS 1%-GIT	Pronasa/lysozyme
14	Bead beating	SDS 1%-GIT	Pronasa/lysozyme
15	Fast DNA for soil		
16	Fast DNA		

After the cell lysis, all samples were centrifuged at 8000*g* for 10 min at room temperature to remove cells debris as well as the beads. The supernatants containing DNA were precipitated by the addition of 1/10 vol of 3 M sodium acetate (pH 5.2) and 2.5 vol of ethyl alcohol and stored overnight at −20 °C. Samples were centrifuged at 8000*g* for 10 min, supernatants were removed and the pellets containing DNA were dried for 10 min in a speed vacuum centrifuge and resuspended in 30 μl of ultrapure water.

(ii) Mechanical lysis-phenol extraction: After each of the cell lysis treatments (sonication, freezing/thawing and bead beating) supernatants containing DNA were centrifuged at 12,000 rpm for 10 min at room temperature to remove cells debris as well as the beads, and transferred into sterile 1.5 ml tubes. DNA was extracted with 1 vol of phenol, followed by a second extraction with 1/2 vol phenol–1/2 vol chloroform:isoamyl alcohol (24:1). DNA was precipitated in the aqueous phase by addition of 2.5 vol of ethyl alcohol overnight at −20 °C. Samples were then centrifuged at 8000*g* for 10 min, the supernatants were removed and the pellets containing DNA were dried for 10 min in a speed vacuum centrifuge and resuspended in 30 μl of ultrapure water (methods 4–6).

(iii) Mechanical lysis in the presence of SDS–chemical extractants–phenol extraction: Methods 7–10 were similar to the phenolic extraction methods described above but including a previous treatment with hexadecylmethylammonium bromide (CTAB) or guanidine isothiocyanate (GIT) and 1% SDS.

- Methods 7 and 8, CTAB treatment. About 100 mg wet/weight cells were resuspended in 1 ml of lysis buffer with 1% SDS. After the lysis treatment (sonication or bead beating), samples were incubated with 200 μl of NaCl 5 M and 150 μl of CTAB for 1 h at 65 °C. Then, cells were frozen for 2 min in liquid N_2 and placed in a bath at 65 °C. DNA was extracted with phenol as described previously.
- Methods 9 and 10, GIT treatment. These methods were similar to methods 7 and 8 but included treatment with guanidine isothiocyanate (GIT) 5 M, EDTA 0.1 M and sarkosyl L-laurylsarcosina 0.5%) for 15–30 min at 60 °C, followed by incubation with ammonium acetate 7.4 M for 15 min at 60 °C. DNA was extracted with phenol as described above.

(iv) Mechanical lysis in the presence of SDS–enzymatic incubation–chemical extractants–phenol extraction: methods 11–14 were similar to methods 7–10 but included enzymatic incubations with lysozyme and pronase prior to treatment with CTAB or GI. About 100 mg wet/weight cells were used. After cell lysis, samples were incubated with 30 μl of lysozyme (300 mg/ml) for 1 h at 37 °C with agitation. Then, 15 μl of pronase (10 mg/ml) and 100 μl of 10% SDS were added and incubated for 1 h at 37°. Treatment with either CTAB or GIT was followed by DNA extraction with phenol as described previously.

(v) Commercial kits: The following commercial DNA extraction kits were used according to the manufacturer's instructions: Fast DNA kit and Fast DNA Spin kit for soil (Bio 101, Carlsbad, CA,

USA) using the mixture of ceramic and silica beads provided in the kit and six pulses of 40 s at a speed of 5.5 on the FastPrep bead beating instrument (Bio 101). The manufacturers did not supply complete information on the composition of the ingredients of their commercial kits. Technical information indicate that lysis buffers were high strength chaotrophic guanidium salts and detergents, with washing buffers consisting of low strength chaotrophic salts, and Tris/alcohol/acid buffers for DNA elution.

In all the methods, integrity of the total DNA was checked by agarose gel electrophoresis. Nucleic acid extracts were stored at −70 °C until further analysis.

DNA quantification

After extraction, DNA concentrations were quantified spectrophotometrically by calculating the A260/A230 and A260/A280 ratios for humic acid and protein content, respectively. A standard curve of DNA concentration (10–50 ng of DNA) was constructed using herring sperm DNA (Sigma Chemical, St. Louis, MO). The DNA yields were estimated on the basis of at least three replicate determinations.

PCR for DGGE analysis

Universal eukaryotic primers targeting 18S rDNA were used for DNA amplification. For DGGE primers Euk20F (5′GTA GTC ATA TGC TTG TCT C 3′) and Euk516r-GC (5′ ACC AGA CTT GCC CTC C 3′), which amplify fragments of about 500 bp [5], were used. These primers were checked against a database of about 4000 eukaryotic sequences with satisfactory results [5]. PCRs were run in a Perking Elmer Cycler in 150 μl tubes using 100 μl reaction volumes. The reaction mixture contained 5 μl of template, 0.5 μl of both primers (50 μM), 1 μl of 25 mM solution of nucleotides, 1 × reaction buffer and 0.5 U of Taq DNA polymerase (Promega Co., Madison, USA). The program included an initial denaturation at 94 °C for 5 min, followed by 19 touchdown cycles of denaturation at 94 °C for 1 min, annealing at 64 °C (with the temperature decreasing 0.5 °C each cycle) for 1 min and extension at 72 °C for 3 min. Final extension was done at 72° for 6 min. Aliquots of 5 μl of the PCR product were run in 1% agarose gel at 130 V for 1 h, stained with ethidium bromide, and quantified using an appropriate standard (*Φ*29 digested with *Hind*III).

DGGE electrophoresis

DGGE was carried out using a DCode Universal Detection System instrument and a gradient former model 475 according to the manufacturer's instructions (Bio-Rad). Electrophoresis was performed with 0.75 mm thick 6% polyacrylamide gels with a ratio of acrilamide to bisacrilamide of 37.5:1, and a denaturing gradient (urea/formamide) from 20% to 60%. The running buffer was TAE (40 mM Tris, 40 mM acetic acid, 1 mM EDTA, pH 7.4). Approximately 100 ng of PCR products were mixed with the same volume of loading dye (2% bromophenol blue, 2% xylene cyanol, 100% glicerol) and applied to individual wells. Gels were run at 60 °C for 11 h at 100 V, stained with ethidium bromide and visualized under UV illumination.

DNA bands were cut from the gel with a sterile blade and placed in sterile vials. Twenty microlitres of milliQ water were added. DNA was allowed to diffuse into the water at 4 ºC overnight. Five millilitres of the eluate were used as a DNA template in a PCR with the primers described above. The PCR products were directly sequenced with a Big-Dye sequencing kit (Applied Biosystem) as described by the manufacturer's instructions. The sequences were aligned to 18S rDNA sequences obtained from the National Center of Biotechnology Information Database using BLAST search.

Clone library construction

18S rDNA genes were amplified from DNA extracted from all environmental samples by PCR using two general eukaryotic primers, Euk1a (5′ CTG GTT GAT CCT GCC AG 3′) and EukBr (5′ TGA TCC TTC TGC AGG TTC ACC TAC 3′) [5]. PCR conditions were as follows, initial denaturation at 97 °C for 5 min, followed by 29 cycles of denaturation at 94 °C for 40 s, annealing at 40 °C for 1 min, and extension at 72 °C for 1 min. The amplification products were used to construct a clone library with the TOPO 1 Kit (InvitroGen, Carlsbad, CA, USA). Positive transformants (white colonies) were streaked for isolation and stored in both stab cultures and dimethyl sulfoxide. Clones were numbered continuously from 1 to 100, digested with the restriction enzyme *Xba*I. Clones with different restriction patterns were sequenced as described above.

To calculate the percentage of similarity between the sequences obtained by using DGGE and cloning, a distance matrix was generated by Neighbor-joining with the correction method of Felsenstein as implemented in ARB, taking into account all unambiguous nucleotides between *Saccharomyces cerevisiae* positions 20–563. The sequences were aligned with 18S rDNA sequences obtained from the National Center of Biotechnology

Information Database and checked for potential quimeras with the Bellerophon Chimera Check program. The sequences were subsequently aligned with 18S rDNA reference sequences in the ARB package (http://www.arb-home.de). The rRNA alignments were corrected manually and alignment uncertainties were omitted. Only unambiguously aligned positions were used to calculate the similarity coefficients.

Results

Comparison of DNA extraction procedures using natural samples

When different DNA extraction procedures were assayed with Río Tinto samples, all except procedures 1–3 yielded ethidium bromide-stained bands in agarose gels. Bead-beating, sonication and freezing/thawing cycles failed to allow the isolation of any substantial amount of DNA. The size distribution of the DNA fragments (1.3–4.5 kb) obtained by gel electrophoresis with each of the remaining procedures revealed that some limited shearing took place during the extractions regardless of the physical disruption method employed.

The DNA yields varied considerably with the different extraction procedures (Table 2). The best DNA yields were obtained when samples were treated with both commercial DNA extraction kits (63 and 58 μg/g dry weight), followed by the CTAB, GIT and enzymatic treatments (methods 7–14, with a range from 15 to 28 μg/g dry weight), and the lowest when only mechanical cell lysis protocols were used (methods 4–6, with a range from 1 to 10 μg/g dry weight). For each sample, procedures that included bead-mill homogenization yielded larger amounts of DNA than procedures that used sonication. However, no difference was found in the amounts of DNA yielded when enzymatic lysis with pronase was introduced. Similarly, comparing treatments with and without CTAB, GIT and enzymes, no differences were found with respect to the purity of the isolated DNA (measured as A260/A230 ratio). Taking into consideration the low yields of DNA obtained by using the freeze-thawing method, it was discarded for the subsequent experiments.

In summary, bead-mill homogenization was found to be superior to the other mechanical cell lysis methodologies assayed for DNA extraction. Both commercial DNA extraction kits yielded the highest amounts of DNA with highest level of purity.

The cell lysis efficiencies of the different procedures were also estimated by microscopic examination before and after DNA extraction (Table 2). The extent of cellular disruption varied between 9% and 99%. All of the procedures, even the ones from which no DNA was recovered, resulted in considerable cellular lysis (more than 60%) except for the freezing–thawing method with less than 10% of broken cells. Although bead-mill homogenization resulted in greater cell disruption than sonication, this effect by itself cannot explain the significantly greater DNA yield obtained with this treatment (compared lanes 4 and 6 in Table 2). Probably the vigorous shaking during bead-beating resulted in the liberation of more DNA from lysed cells into the extraction mixture. As expected, there was a strong

Table 2. Efficacy of DNA extraction procedures evaluated on the basis of DNA yield and cell lysis

Method	Treatment	DNA yields (μg/g dry wt of sample)	$A_{260/230}$	$A_{260/280}$	% Cell lysis
1	S	<0.1			70±4
2	F	<0.1			10±3
3	B	<0.1			95±6
4	S/Ph	6.3±1	1.10±0.03	1.72±0.03	78±6
5	F/Ph	1.0±0.3	1.20±0.04	1.91±0.06	9±2
6	B/Ph	10±3	1.22±0.08	1.95±0.03	94±5
7	S/SDS/CTAB	17±4	0.91±0.09	0.99±0.06	61±9
8	B/SDS/CTAB	22±2	1.10±0.06	1.20±0.08	98±3
9	S/SDS/GIT	15±6	1.34±0.02	0.97±0.05	68±9
10	B/SDS/GIT	25±7	1.11±0.09	1.11±0.08	95±5
11	S/SDS/CTAB/PL	21±6	0.99±0.10	1.24±0.03	58±11
12	B/SDS/CTAB/PL	23±1	1.22±0.04	1.14±0.08	99±6
13	S/SDS/GIT/PL	28±5	1.03±0.08	0.97±0.04	71±7
14	B/SDS/GIT/PL	15±6	1.50±0.02	1.50±0.06	97±3
15	Fast DNA soil	63±8	0.98±0.03	1.28±0.05	99±7
16	Fast DNA	58±3	1.21±0.08	1.12±0.08	98±2

S—sonication, F—freezing–thawing, B—bead beating, Ph—phenolization, PL—pronasa-lysozyme.

positive correlation between DNA yields and extent of cellular lysis ($r = 0.85$, data not shown) for the procedures that yielded significant amounts of DNA.

Impact of DNA isolation method on DGGE analysis

The DNAs extracted using the different methods compared in this work were suitable as PCR templates. We obtained PCR products in all cases except with mechanically disrupted cells (methods 1–3), where no PCR products were achieved. DGGE performance was further tested with DNA obtained from methods 4–16 once the optimal conditions for electrophoresis were defined. However, since DGGE results obtained for methods involving sonication/extractants (methods 7 and 9) and sonication/enzymes/extractans (methods 11 and 13) were always very poor, the respective gels are not shown. The PCR products were run in DGGE to identify the eukaryotes present in the samples (Fig. 1). Since similar results were obtained for the six samples analysed Fig. 1 shows only the DGGE gels obtained for RT-40 (Fig. 1a) and RT-56 (Fig. 1b). The number of bands yielded by each DNA extraction method is summarized in Fig. 1c.

Although significant differences between the DNA isolation methods were observed, the community profiles obtained by the different methods within a

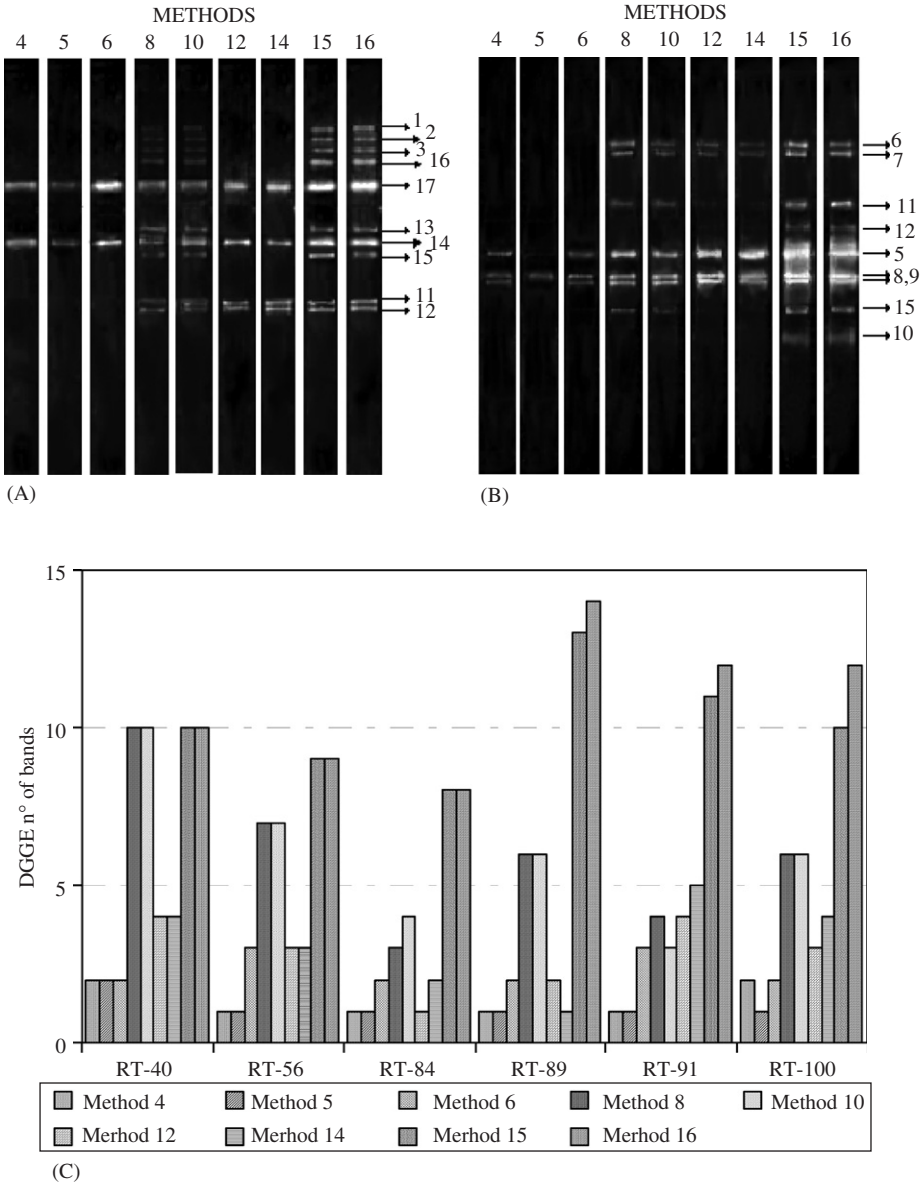

Fig. 1. DGGE gel separation of PCR-amplified 18S rDNA fragments: (A) sample RT-40, (B) sample RT-56. Lane numbers correspond to the DNA extraction methods. Arrows correspond to the bands identified in Table 4. (C) Number of DGGE bands obtained per DNA extraction method assayed.

given sample, resembled each other (Fig. 1a, b). For all the samples analysed, extraction methods 4–6 revealed fewer bands than those methods that received additional treatments with detergents, chemical extractants and enzymes (Fig. 1c). Although the addition of extractants such as GIT or CTAB resulted in a higher number of bands (methods 8 and 10), the additional use of enzymes resulted in a decrease in the number of bands (methods 12 and 14). Commercial kits , methods 15 and 16, yielded the broadest and the most intense band patterns. Three of the six samples (RT-40, RT-56 and RT-84) did not show differences between the number of bands of both methods. However, method 16 yielded a higher number of bands for the remaining samples than the DNA extraction method 15. For this reason, all the molecular analysis of the samples were carried out using only DNA extracted by this methodology.

Identification of eukaryotic populations

Table 3 summarized the species identified by using optical microscopy, DGGE and 18S cloning as well as their relative abundances per sample.

Microscopic analysis

Seventeen species belonging to different genera were identified based on their morphological characteristics and previous studies of the eukaryotic communities in this river [1,14,15]. One species of diatoms related to the genera *Pinnularia*; one species of euglenas, *Euglena mutabilis*; five species of green algae, *Chlamydomonas* spp., *Chlorella* sp. and *Dunaliella bardawil Stichococcus* sp. and *Mesotaenium kramstai*; two species of filamentous green algae *Zygnemopsis* sp. and *Klebsormidium* sp.; one species of amoeba belonging to the genera *Vahlkampfia*; one species of heliozoan, *Actinophrys* sp.; four species of flagellates related to the genera *Bodo, Cercomonas, Ochroomonas* and *Labirynthula*; one species of ciliates, *Oxytricha sp.* and one species of rotifer *Rotaria* sp.

Direct microscopic counts were used to estimate total biomass abundances and diversity in each sample (Table 3). The results revealed that each sample was constituted of a virtually monospecific population, i.e. *Zygnemopsis* sp. represent 75% of the cell abundances in sample RT-40, *Pinnularia* was the dominant species in RT-89 with 61% of the total cell number, and *Euglena mutabilis* showed 53% of the total cell biomass in RT-100. In general, green algae and filamentous algae were mainly responsible for the total eukaryotic biomass present in these samples. However, heterotrophic species such as ciliates, amoebae, rotifers or flagellates usually represent less than 5% of the total biomass.

DGGE analysis

A total of 65 bands were identified in the six environmental samples analysed using DGGE (Table 4). For all the samples analysed, BLAST search yielded a smaller number of taxa than the morphological analysis (Table 3). In the same manner, all the taxa identified by DGEE were confirmed by microscopic observations except for *Chlamydomonas*. In this case,

Table 3. Species observed and relative cell abundances determined by using three techniques: (M) Optical microscopy, percentages of species abundances, (D) DGGE, percentage of number of bands per species identified, (C) 18S rDNA gene cloning, percentage of number of clones obtained per species

		RT-40			RT-56			RT-84			RT-89			RT-91			RT-100		
		M	D	C	M	D	C	M	D	C	M	D	C	M	D	C	M	D	C
Filamentous algae	*Zygnemopsis* sp.	75	40	50	—	—	—	12	—	—	—	—	—	62	33	53	—	—	—
	K. flaccidum	—	—	—	—	—	—	40	25	11	—	—	—	12	25	12	—	—	—
Green algae	*D. bardawil*	10	30	5	—	—	—	—	—	—	—	—	—	—	—	—	9	35	50
	Chlamydomonas spp.	8	20	35	20	11	28	17	50	66	7	21	53	—	—	—	—	—	—
	Chlorella sp.	—	—	—	31	33	39	—	—	—	10	—	9	9	17	29	7	16	11
	Stichococcus sp.	—	—	—	5	—	—	—	—	—	7	7	—	—	—	—	7	—	—
	M. kramstai	—	—	—	8	—	—	—	—	—	—	—	—	—	—	—	9	8	—
Diatoms	*P.* cf. *interrupta*	—	—	—	9	—	—	—	—	—	61	44	29	—	—	—	6	25	28
Euglenas	*E. mutabilis*	—	—	—	14	—	—	—	—	—	—	—	—	—	—	—	53	—	—
Ciliates	*O. granulifera*	1	—	—	3	22	10	—	—	—	6	14	2	—	—	—	—	—	—
Kinetoplastida	*Bodo* sp.	—	—	—	4	12	6	—	—	—	—	—	—	—	—	—	6	16	6
Amoebae	*Valkhampfia* sp.	—	—	—	—	—	—	11	—	2	2	—	—	2	—	—	—	—	—
Rotifers	*Rotaria* sp.	—	—	—	—	—	—	—	—	—	—	—	—	3	—		—	—	—
Heliozoa	*Actinophyrs* sp.	—	—	—	—	—	—	4	—	—	—	—	—	—	—	—	—	—	—
Flagellates[a]		1	—	—	2	—	—	8	—	1	3	—	—	5	—	2	3	—	5
Fungi[b]		5	10	10	4	22	17	8	25	20	4	14	7	7	25	4	—	—	—

[a]Flagellates includes *Cercomonas* sp., *Ochroomonas* sp. and *Labirynthula* sp.
[b]Fungi includes S. *polyschides*, *Phialophora* sp., and R. *longisetosum*.

Table 4. Identity of DGGE excised eukaryotic bands

Sample	Bands	Closest relative	% Similarity	Taxonomic group
RT-40	1,2,3,16	*Zygnemopsis* sp.	86.2	*Streptophyta*
	11	*Siphula polyschides*	45.0	*Ascomycota*
	12	*Chaetosporadinium ovalis*	61.5	*Colcochaetales*
	13,14,15	*Dunaliella bardawil*	90.2	*Chlorophyta*
	17	*Chlamydomonas noctigama*	96.1	*Chlorophyta*
RT-56	5,6,7	*Chlorella* sp.	97.1	Chlorophyta
	8,9	*Oxytricha granulifera*	92.8	*Ciliophora*
	10	*Bodo* sp.	55.0	*Kinetoplastida*
	11,12	*Phialophora* sp.	94.6	*Ascomycota*
	15	*Chlamydomonas noctigama*	91.4	*Chlorophyta*
RT-84	1,2,3,4	*Chlamydomonas pitschmannii*	95.6	*Chlorophyta*
	5,8	*Klebsormidium flaccidum*	91.4	*Klebsormidiales*
	11,12	*Raciborskiomyces longisetosum*	98.1	*Ascomycota*
RT-89	1,3,4	*Chlamydomonas pitschmannii*	94.7	*Chlorophyta*
	6,8,10,11	*Pinnularia* cf. *Interrupta*	90.5	*Bacillariophyta*
	13,14	*Chlorella* sp.	98.1	*Chlorophyta*
	15,16	*Raciborskiomyces longisetosum*	98.5	*Ascomycota*
	21	*Stichococcus* sp.	94.7	*Microthamniales*
	24,28	*Oxytricha granulifera*	93.8	*Ciliophora*
RT-91	2,4,5	*Klebsormidium flaccidum*	95.2	*Klebsormidiales*
	8,9,11,13	*Zygnemopsis* sp.	91.4	*Streptophyta*
	10,14	*Chlorella* sp.	98.4	*Chlorophyta*
	22,23,27	*Raciborskiomyces longisetosum*	96.9	*Ascomycota*
RT-100	2,3,5,7	*Dunaliella bardawil*	91.1	*Chlorophyta*
	9,11	*Chlorella* sp.	94.8	*Chlorophyta*
	10,14,15	*Pinnularia* cf. *Interrupta*	91.6	*Bacillariophyta*
	18	*Mesotaenium kramstai*	97.5	*Chlorophyta*
	22,24	*Bodo* sp.	69.7	*Kinetoplastida*

seven of the obtained sequences related to *Chlamydomonas* were assigned to *C. pitschmannii* (ca. 95% of sequence similarity) and two sequences were related to *C. noctigama* (ca. 93% of similarity). Neither species was distinguished previously by microscopy. On the contrary, some species located by microscopy in these samples were never detected by DGGE, e.g. euglenoids, heliozoa, amoeba or rotifers, even though, in the case of euglenoids they constituted one of the dominant species in the sample.

Additionally, several sets of bands having different migration positions within a given sample yielded the same species identification (i.e. sample RT-40, bands 1-2-3-16 were all related to *Zignemopsis* sp., Table 4). In fact, only four of the identified species were detected by unique bands (*S. polyschides*, *C. noctigama*, *Stichococcus* sp. and *M. kramstai*). Furthermore, the intensity of the bands did not correspond to the relative species abundance for most of the cases, i.e. the most intense bands in the profiles obtained in sample RT-40 corresponded to *D. bardawil* and *C. noctigama* (bands 14 and 17, respectively in Fig. 1a and Table 4) where the dominant species in this sample was *Zygnemopsis* sp. with the 75% of the total cell biomass (Table 3).

18S rDNA clone library

The cloning reactions for each sample produced ca. 100 clones that were sequenced. As had occurred with the DGGE, the BLAST search yielded a smaller number of species than the number of organisms identified morphologically and all the species identified by cloning were also confirmed by microscopy. All the sequences related to *Chlamydomonas* formed two distinct clusters closely related to both *Chlamydomonas* species identified by DGGE, *C. noctigama* and *C. pitschmannii*, which confirmed the presence of these species in the samples.

Likewise in the case of the DGGE, the percentage of clones per species usually did not reflect their relative abundance in the sample. Thus, green unicellular algae such as *Chlamydomonas* spp. (RT-40, RT-84, RT-89 and RT-91) or *Chlorella* sp. (RT-91), showed a higher percentages of clones than expected given their relative

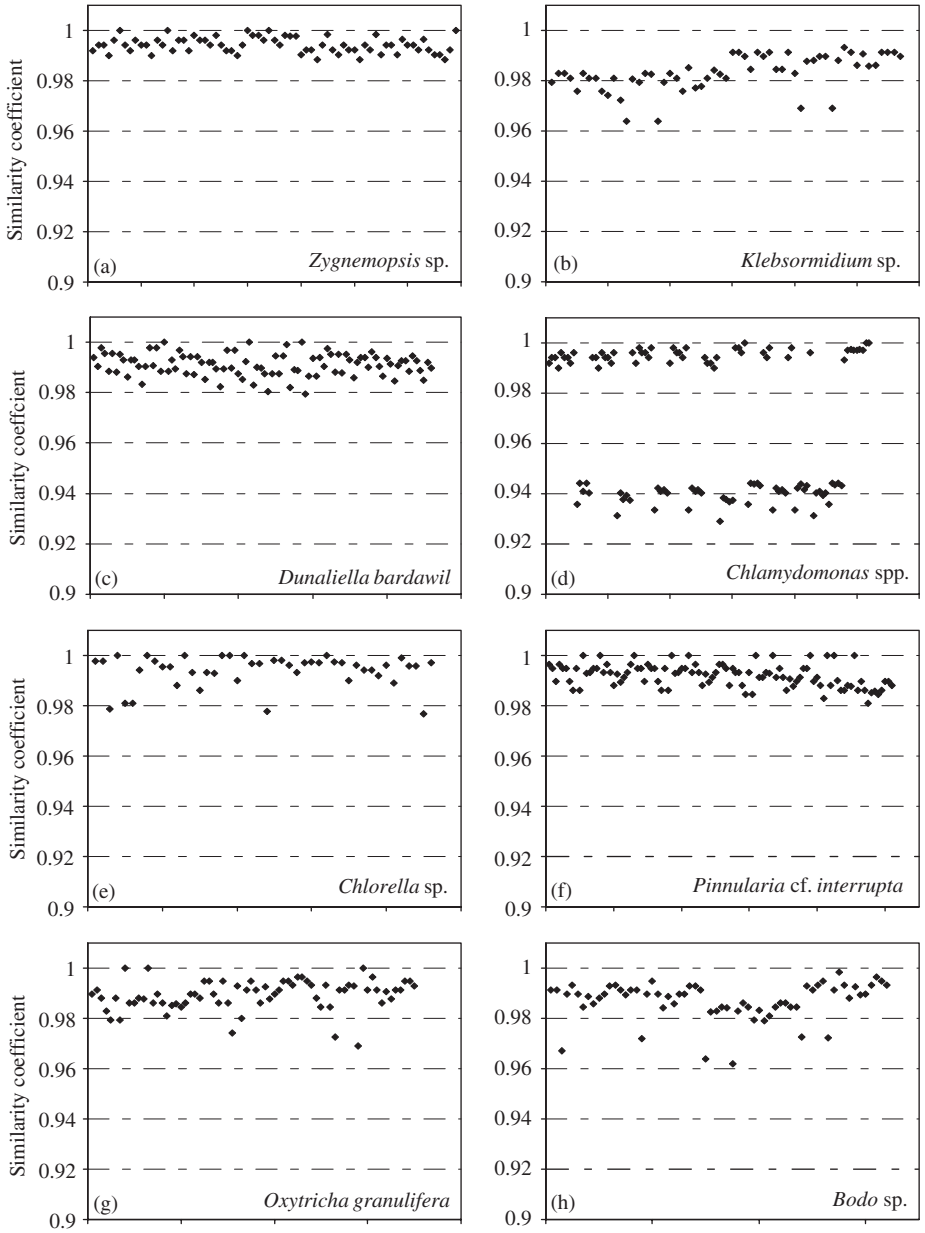

Fig. 2. Coefficients of similarity between 18S rRNA sequences obtained from the distance matrix generated by the Neighbor-joining method.

biomass in the samples. In this case, there were also missing organisms such as euglenoids, green algae (*Stichococcus* sp. and *M. kramstai*), rotifers and heliozoan.

All the sequences obtained by DGGE and cloning were compared in a distance matrix and the similarity coefficients are summarized in Fig. 2. The overall similarity of the sequences from the same species was independent of the methodology used, showing similar coefficients between 0.98 and 1 (Fig. 2a, c, e, f). However, a discrete higher variation among sequences was found in species related to *K. flaccidum*, *O. granulifera* and *Bodo* sp. (Fig. 2b, g and h, respectively) where the similarity coefficients ranged from 0.96 to 1. The coefficients obtained from *Chlamydomonas* sequences (Fig. 2d) were clearly spread in two different sets, confirming the presence of two different species.

Discussion

The main objective of this work was to compare the efficiency of DGGE in the study of the eukaryotic community present in an acidic environment in an attempt to find an accurate and reliable methodology

that would be easier and less time consuming than conventional microscopy approaches. We can divide this work into two parts, (i) the evaluation of different protocols for eukaryotic DNA extraction, and (ii) the comparative analysis of eukaryotic diversity using different molecular techniques.

DNA extraction methods

The development of efficient and comparative DNA extraction methods is one of the most critical steps for evaluation of the microbial diversity of a given environment using molecular approaches. The study of sediments further complicates DNA extraction due to the need to separate DNA from substances interfering with its analysis, e.g. heavy metals [40]. Our aim was to optimize DNA isolation for community analysis in sediments from acidophilic environments. Even though commercial methods (15 and 16), Fast DNA extraction kit for soil and Fast DNA extraction kit, yielded DNA of large molecular size with almost no fragmentation, two methods, 8 and 10 (cells disrupted by bead beating and treatment with CTAB or GIT) gave DNA of similar quality, in terms of intactness and amplification efficiency, although with one third lower efficiency than the commercial kits. The addition of an enzymatic treatment (pronase and lysozyme) did not improve the efficiency of amplification, on the contrary, fewer bands were obtained when analysed by DGGE. DNA fragmentation increased when chemical and enzymatic treatments were introduced.

Our findings support the results of previous studies [12,13] where the use of bead beating for an efficient cell lysis for the extraction of DNA were recommended. In these studies hot detergent treatment and freeze-thaw cycles were observed to be rather ineffective in disrupting cells. Our results concur, with percentages of cell lysis ca. 98% for the bead beating system and less than 10% when cells were treated with freeze-thaw cycles. Although sonication produces a high percentage of cell lysis (ca. 70%), DNA fragmentation was greater than with beads. In the same manner, higher DNA yields were obtained with bead mill homogenization than with the other two physical treatments (sonication and freeze-thawing cycles).

Although CTAB has been used in previous studies to remove contaminants from DNA [2,8], none of the purification agents tested (CTAB and GIT) completely removed all the contaminants present in the samples. Additional purification steps involving CTAB or GIT yielded purification efficiencies equivalent to the rest of the extraction protocols.

In our case, DNA recovery was higher when commercial kits were used, three times more than the DNA yields obtained with the second best methods, 10 and 14, which also include bead beating and GIT (60 and 20 μg/g dry wt, respectively). Although technical information supplied with the commercial kits mention that lysis buffers contain high-strength guanidium salts and detergents, without knowledge of the exact components in each of the commercial kits the reasons for their apparent advantage can only be speculative.

The use of DGGE with universal primers reveals dominant species [20], however when rare species are studied more gentle or harsher DNA isolation methods may be optimal, depending on cell envelope structure [40]. Krsek and Wellington [12] compared a variety of treatments in the isolation and purification of DNA from soil using DGGE in the analysis of one soil sample. They observed that DNA extracts obtained with different procedures yielded patterns with differences in intensities and number of bands and recommended a combination of bead-beating with lysozyme and SDS followed by a phenol/chloroform purification and isopropanol precipitation. Our results support these findings regarding the efficiency of the combined action of detergent, lysozyme and bead-beating, although in our samples, shearing of DNA was extensive with the methods including detergent and enzyme treatment, and the number of bands yielded in the DGGE by these protocols were lower in number and intensity than those obtained with methods that did not include these treatments (methods 8, 10 or 14, 15).

Clear conclusions can be drawn from our results. Each isolation and purification method yielded distinctly different DGGE profiles. Methods that include bead-beating as well as chemical extractants and commercial kits recovered the broadest spectrum of eukaryotes and were the easiest to perform.

Analysis of the extracted DNA

Although molecular techniques, such as DGGE, have been proven particularly useful for an initial investigation to distinguish and identify the dominant community members, the interpretation of data for eukaryotic diversity study in natural environments presents uncertainties, and several difficulties may arise in the long processes required for natural samples to sequence [6,31]. Some of the problems are intrinsic to PCR amplification kinetics, and, therefore, are shared by all of the approaches that use this step (PCR cloning and PCR fingerprinting techniques). Other difficulties are specific for the DGGE technique used in this study. We used independently obtained microscopy-based information for some of the organisms present in Río Tinto to investigate the magnitude of the difficulties.

One of the main problems we found when DGGE is used was the presence, in the same sample, of more than one band showing different mobility but the same

identity (Table 4). This could be due to the formation of heteroduplex molecules during the amplification process that might contribute to difficulties in the interpretation of community complexity from DGGE patterns [7,21,32]. DGGE analysis of two PCR products after inducing heteroduplex formation will result in four bands, two heteroduplex and two homoduplex molecules, leading to an overestimation of the real number of community constituents [10,29]. However, when we excised, reamplified, reelectrophoresed and sequenced all of these bands, only two of them (10%) yielded multiple bands. The multiple bands (presumably representing homo- and heteroduplexes) were clearly separated in the gel when we performed a new DGGE analysis, and, therefore, they cannot be related to the quality of gel separation or the precision of the excision. These results agree with previous studies reported by Murray et al. [17].

An important result of this study was the observation that some of the species detected by microscopy were not detected by the molecular analysis. Most of the heterotrophic species such as heliozoan, amoebae or rotifers detected by microscopy, were not molecularly identified and, more surprisingly, species as abundant as *Euglenas* was never detected by DGGE or cloning. In the same manner, the band intensity or the number of clones retrieved per species did not always correlate with the abundance observed microscopically. Thus, members of the *Chlorophyta* such *Chlamydomonas*, *Chlorellas* or *Dunaliellas* appeared to be relatively easily amplified in our study regardless of their abundance in the samples, producing the most intense bands in the DGGE.

This fact could be due to the practical problems inherent to both molecular methodologies used: that the relative abundance of any population detected may deviate from that actually present in a sample. Although, in principle, procedures for extracting DNA may be selective, the primer designs could compromise PCR amplification. Thus, using primers targeting plastid genes, rather than the primers specific for eukaryotes, may improve the detection of elusive species [29]. Furthermore, the relative large genome and potentially high *rrn* copy number of species related to Chlorophyatas, such as *Chlamydomonas*, may partially explain the apparent preferential amplification and detection of these species in complex environmental samples [11,24].

In the same manner, despite DNA extraction and PCR biases, it has been demonstrated that is not always possible to separate DNA fragments which have a certain amount of sequence variation [4,35] and we cannot eliminate the possibility that some of the minor bands might be masked if they exhibited the same melting behaviour as other populations or that their concentrations were less than the detection limit of the staining solution. More information might be obtained from weak bands by employing a more sensitive gel developing protocol such silver staining. Although some work has been carried out in this regard [23], the limitations of currently available methodologies lead many authors to conclude that the ecological quantification of organism abundances based on molecular approaches have not yet been convincingly reported [25,31,38].

Nevertheless, one of the strongest points of the application of DGGE in microbial ecology is the possibility of performing simultaneous analysis of multiple samples as well as the advantage of obtaining bands that can be sequenced and compared. Eukaryotes have been traditionally identified based on morphological characteristics using microscopy-related techniques. These methodologies are time-consuming, require expertise in taxonomy and, sometimes, fail to distinguish among similar species. Although sequences obtained from DGGE are short (ca. 500 pb), the reliability of the DGGE regarding taxonomic identification is high, at least at the genus level. In this regard, two sequences corresponding to different species of *Chlamydomonas* have been detected in this work using molecular techniques, species that were not previously distinguished microscopically due to their similar morphology.

In conclusion, we think that the application of DGGE techniques could be as useful for eukaryotic communities as it is for prokaryotic ones. However, our results also illustrate that any method, even a molecular one, only reflects a portion of the real biodiversity. Only an integrated approach combining molecular techniques, microscopic observations and new isolation strategies will guarantee a more realistic picture of the microbial diversity of any given ecosystem.

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