RESEARCH ARTICLE

High genetic diversity and novelty in planktonic protists inhabiting inland and coastal high salinity water bodies

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Abstract

We analyzed the genetic diversity (18S rRNA gene) of planktonic microbial eukaryotes in 34 different coastal and inland saline ponds. A wide range of environmental conditions was covered with up to 30-fold differences in salinity concentrations (12.5–384 g L^{-1}), and *in situ* temperatures (1.3–37.5 °C), and three orders of magnitude in the trophic status (i.e. chlorophyll $a < 0.1$ to >50 mg L^{-1}). Geographically distant sites were studied with contrasting salt origins, and different temporal patterns of wetting and drying. The genetic diversity was high, far beyond the few groups traditionally considered as high salinity-adapted, with sequences spread throughout eight high-rank taxonomic groups and 27 eukaryal classes. The novelty level was extremely high, with 10% of the whole dataset showing < 90% identity to any previously reported sequence in GenBank. Opisthokonta and Rhizaria contained the highest novelty and Chlorophyta and Alveolata the lowest. Low identity sequences were observed both in coastal and inland sites and at lower and at higher salinities, although the degree of novelty was higher in the hypersaline waters (> 6.5% salinity). Overall, this study shows important gaps in the current knowledge about protists inhabiting continental (hyper)saline water bodies, highlighting the need for future, more detailed investigations.

Introduction

Salinity is one of the most important factors globally selecting and structuring microbial assemblages (Lozupone & Knight, 2007; Auguet *et al.*, 2010; Barberán & Casamayor, 2010), and several salinity stress adaptation strategies have been developed in microorganisms inhabiting high salinity environments, mostly in prokaryotes (Oren, 2002). It has been suggested that eukaryotes have greater difficulties coping with the selective effect of high salinity (Pedrós-Alió *et al.*, 2000; Oren, 2002), resulting in large decreases in the number of species as salinity increases (Hauer & Rogerson, 2005). This thinking has led to the belief that eukaryotes are a poorly represented domain in high salinity environments as compared with prokaryotes. Traditionally, only a few species have been considered adapted to high saline stress, such as the ubiquitous *Dunaliella salina* and several ciliates commonly found in hypersaline waters (e.g. *Fabrea salina*, *Euplotes* spp.), and a few types of diatoms (Oren, 2005). Halophile and halotolerant melanized fungi, ('black yeasts', *Ascomycota*), have also been found to be present in high abundance and diversity in solar salterns (Gunde-Cimerman *et al.*, 2000). Black yeasts, but also filamentous fungi, have been detected in hypersaline environments elsewhere (Buchalo *et al.*, 1998; Butinar *et al.*, 2005). More recently, a heterolobosean flagellate has been described in saturated brines worldwide (Park *et al.*, 2007), as well as active halophilic stramenopiles, with the description of new bicosoecids such as *Halocafeteria seosinensis* (Park *et al.*, 2006) and *Placididea* (Park & Simpson, 2010), and ciliates of the genus *Trimyema* (Cho *et al.*, 2008). Recently, scuticociliate and oligohymenophorean morphotypes have been reported in deep sea hypersaline environments (Orsi *et al.*, 2012).

Studies analyzing the genetic diversity of the whole eukaryotic assemblages in high salt environments are very scarce, although consistent changes in eukaryotic community composition and richness have been observed along salinity gradients (Casamayor *et al.*, 2002). A study in high mountain saline lakes of the Eastern Tibet Plateau has shown that most of the sequences are affiliated with

Chlorophyta, Dinophyceae and *Ciliophora* (Wu *et al.*, 2009), whereas rich eukaryotic assemblages have been detected in deep hypersaline anoxic basins (Alexander *et al.*, 2009; Edgcomb *et al.*, 2009; Stock *et al.*, 2012). Finally, a genetic fingerprinting analysis of OTUs along the salinity gradient in a multipond solar saltern showed greater richness in the eukaryal assemblages than in prokaryotes, especially at salinities < 110 g L^{-1}, and richness in the same range for prokaryotes and eukaryotes at salinities > 150 g L^{-1} (Casamayor *et al.*, 2002). Overall, from the limited number of studies available it can be hypothesized that (1) a much larger number of eukaryotic species than previously expected may be adapted to high saline stress; (2) environments with high concentrations of salt may hold protists distantly related to any previously known species; and (3) highly novel protists may show differential distributions along the salinity gradient.

In the present work, we have analyzed the genetic diversity of planktonic microbial eukaryotes (size range 0.2–40 μm) along a salinity gradient in 34 different coastal and inland saline water bodies using 18S rRNA gene sequencing of denaturing gradient gel electrophoresis (DGGE) excised bands. Samples were obtained from different geographic regions and covered a wide range of environmental conditions such as salinity (concentration and composition), *in situ* temperatures, trophic status, water and connectivity regimes, and altitude, which captured part of the high variety of saline habitats present in continental areas. We aimed to provide a preliminary view on the eukaryal assemblages inhabiting these environments, focusing on the most abundant populations recovered from a genetic fingerprinting and sequencing analysis using universal PCR primers for the *Eukarya* domain. The study showed a much greater number of eukaryotic phylotypes than previously expected, some of them distantly related to some previously known species (< 90% identity).

Materials and methods

Study sites and sampling

We surveyed 34 sites from different inland and coastal environments (Supporting Information, Table S1). The selected saline shallow ponds represented a wide range of basic ecological and limnological characteristics, such as salt concentration, habitat range (semi-arid to arid inland endorheic regions, and coastal man-made salterns), different hydrologic regimes (permanent and temporal ponds), and connectivity (isolated ponds and connected solar salterns pools). Samples were obtained from different field expeditions (Pedrós-Alió *et al.*, 2000; Demergasso *et al.*, 2004; Estrada *et al.*, 2004). Additional information can be found in Herrero & Castañeda (2009).

Sampling was carried out with a bucket fixed to the end of a pole avoiding both sediment resuspension and collecting samples close to the ends of the ponds. Salinity was measured with a hand-held refractometer (Atago S-28E, Japan), and chlorophyll *a* by fluorescence on acetone extracts as reported (Demergasso *et al.*, 2008). For DNA analyses, water samples were pre-filtered *in situ* through a 40-μm pore-size net, to retain large zooplankton and algae, and 300–500 mL were subsequently filtered on 0.2-μm pore polycarbonate membranes (47 mm diameter). The membranes were stored in lysis buffer (40 mM EDTA, 50 mM Tris, pH 8.3, 0.75 M sucrose), enzymatically digested, and phenol extracted and purified (Hervàs *et al.*, 2009).

The molecular methodology used in this initial survey was based on DGGE separation of 18S rRNA gene segments amplified by PCR, and direct sequencing of excised bands. PCR amplification was run with 'universal' eukaryal primers 1Af (5′-CTGGTTGATCCTGCCAG-3′) and 516r-GC (5′-ACCAGACTTGCCCTCC-3′) with an attached GC clamp (Díez *et al.*, 2001). DGGE was carried out as previously described (Díez *et al.*, 2001) in a denaturant gradient from 40 to 65% (100% denaturant is 7 M urea and 40% formamide). DGGE gels were stained with a solution of GelStar (1 : 5000 dilution; FMC BioProducts) and the most prominent bands visualized under UV radiation were excised, reamplified and directly sequenced (Casamayor *et al.*, 2001). Sequencing was carried out using external facilities (http://www.macrogen.com).

DNA sequences analyses

Initially, the 18S rRNA gene sequences were manually inspected for sequencing errors with BIOEDIT (Hall, 1999) and checked for chimera detection with UCHIME (Edgar *et al.*, 2011) Sequences were further processed with MOTHUR v1.12.0 (Schloss *et al.*, 2009). After BLAST search, sequences matching *Metazoa* were eliminated. Overall, 73 refined 18S rRNA gene sequences were automatically aligned with SINA in SILVA (Pruesse *et al.*, 2012) and imported into the SSU Ref NR 108 database (Pruesse *et al.*, 2007) in ARB (Ludwig *et al.*, 2004). Partial sequences (mean length 470 bp) were inserted in the optimized ARB tree, keeping the overall tree topology by using the parsimony interactive tool.

We explored the 18S rRNA gene novelty of the dataset by BLAST identity search against GenBank sequences (search May 2012). The identity of each single sequence was related to both the closest environmental match (CEM), and the closest cultured match (CCM) available in GenBank. Histograms and dispersion plots (Del Campo & Massana, 2011; Massana *et al.*, 2011) were used to assess the degree of novelty comparing both habitat type

(coastal vs. inland) and salinity gradient (brackish-saline vs. hypersaline).

Faith's phylogenetic diversity (PD) and phylogenetic species variability (PSV) indices were calculated in R (http://www.r-project.org/) with the ape and picante packages. PD was calculated as the sum of the branch length associated with the OTUs from a defined community (Faith, 1992). To standardize for unequal sample size across samples, the mean PD of 1000 randomized subsets was calculated (Barberán & Casamayor, 2010). PSV quantifies how phylogenetic relatedness decreases the variance of a hypothetical neutral trait shared by all species in a community (Helmus *et al.*, 2007). The PSV value is 1 when all species are unrelated (i.e. star phylogeny) and approaches 0 as species become more related.

Nucleotide sequence accession numbers

Sequences were deposited in GenBank with accession numbers AM072914–AM072938, AM084278–AM084326, AM087460–AM087468, AM179805–AM179824 and AM23 1690–AM231700 (details in Table S2).

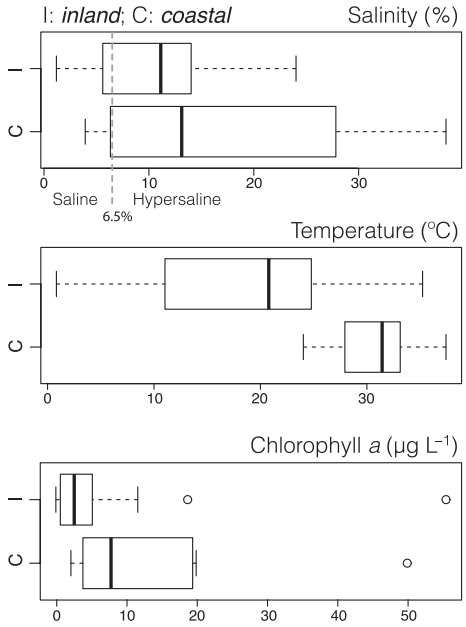

Fig. 1. Boxplots showing the gradients explored for salinity, temperature, and trophic status (Chl *a*) in the 34 sites analyzed. C, coastal saline ponds; In, inland haline water bodies.

Results

The surveyed systems covered a wide range of variability in the environmental conditions such as up to 30-fold differences in both the salinity gradient (from 1.25 to 38.4% salinity) and the *in situ* temperature (from 1.3 to 37.5 °C), and two orders of magnitude in chlorophyll *a* concentrations (Fig. 1, Table S1). In inland waters, salinity ranged between 1.25% (Doline in Atacama, Chile) and 24.1% (Gallocanta lagoon, Spain), whereas in coastal ponds, salinity was 4–38.4% (Santa Pola, Alicante). Most of the samples examined were hyperhalines (i.e. salinity > 6.5%). Colder waters were found in inland sites (range 1–35 °C) than in coastal areas (24–37.5 °C). No significant differences were found in trophic status (i.e. chlorophyll *a* content) for the coastal and inland salt ponds examined (Fig. 1, lower panel).

The 18S rRNA gene sequences obtained were spread along eight high-rank taxonomic groups and matched 27 eukaryal classes (Fig. 2, more details in Table S2 for phylotypes overlapping all 34 samples). Most of the sequences were affiliated with *Viridiplantae* (37%; mainly *Chlorophyta* and *Trebouxiophyceae*), *Alveolata* (30%), *Stramenopiles* (12%), *Opisthokonta* (11%; *Choanoflagellida* and *Fungi*), and *Rhizaria* (5%, cercomonads). *Centroheliozoa, Haptophyceae* and *Telonemida* were less well represented. The genetic novelty of the dataset was high. Between 30% and 40% of the sequences showed an identity match < 97% with any previously reported protist sequence, and *c.* 10% had < 90% identity (Fig. 3, upper panel). Interestingly, such low identity sequences were observed both in coastal and inland sites, and both at low and high salinities (Fig. 3). Thus, any of the saline ponds examined may hold a significant protist novelty. We also observed that the number of phylotypes closer to a previously reported environmental clone was significantly higher in coastal (69%) than in inland sites (56%) (*t*-test, $P < 0.01$), suggesting that more surveying efforts are needed in continental saline waters. The calculated indexes of phylogenetic diversity (PD and PSV) also indicated a larger genetic diversity in inland than in coastal waters (Table 1). Finally, we carried out a pairwise comparison of the phylotypes of each major eukaryal group to compare the identity level between the sequences found in both environments (Table S3). Interestingly, in a few cases (e.g. *Dunaliellaceae, Climascostomidae*) the identities were > 99%.

The novelty level in each eukaryal class was studied by combining dispersion plots of the CEM and the CCM available in GenBank, after examining the averaged identity values within each taxon (Table S2). We split the results according to both habitat type (coastal vs. inland, Fig. 4), and salinity concentration (i.e. saline vs. hypersaline, Fig. 5). For each plot we defined 'the highest novelty plot area' as the area of the plot that contained phylotypes matching < 97% identity to both CEM and CCM. Overlapping this area, we highlighted two additional regions in the plot, the 'cultured gap plot area', for those eukaryal classes that on average showed > 97% with CEM

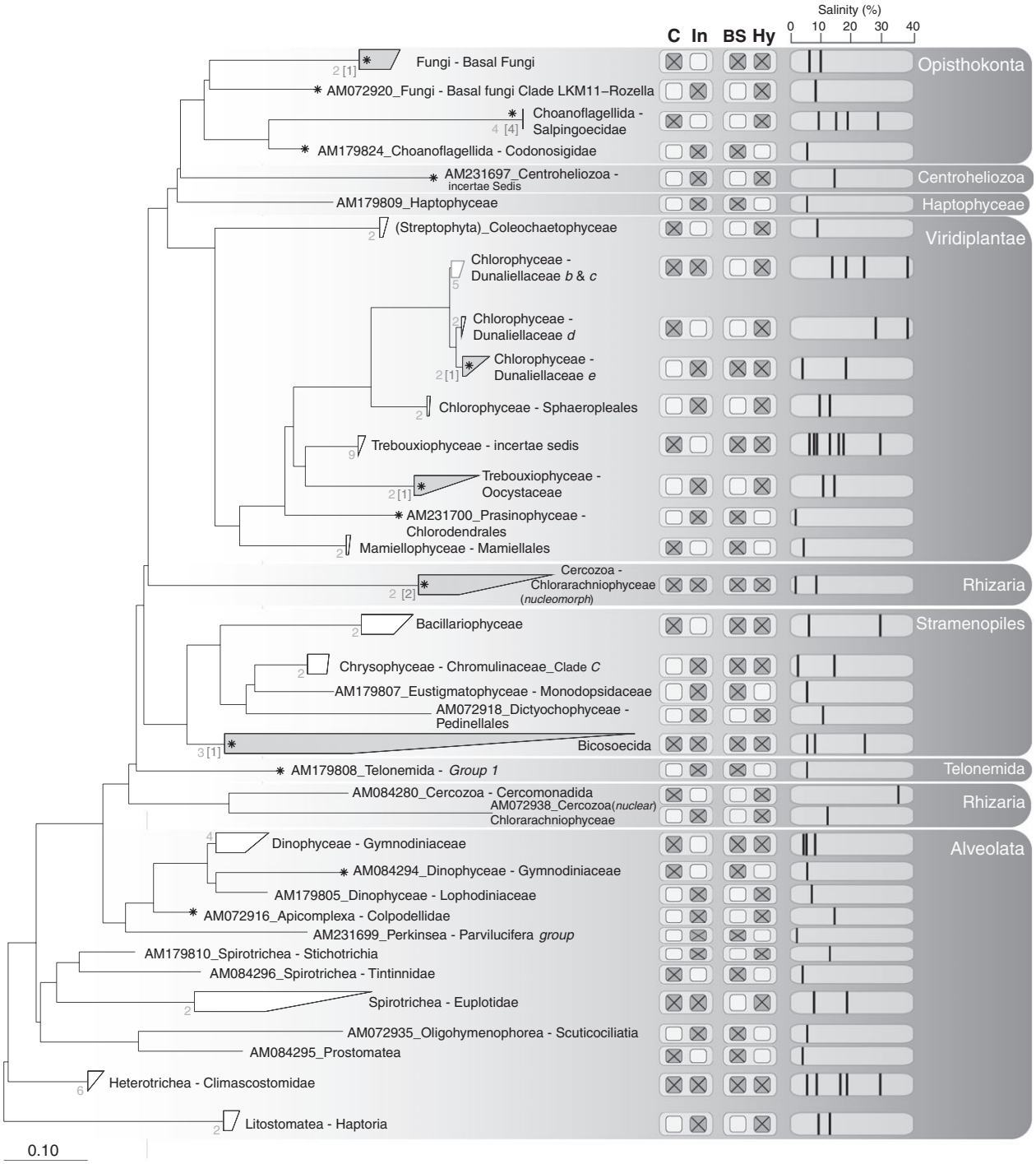

Fig. 2. Collapsed phylogenetic tree for the 18S rRNA gene sequences obtained from the different water bodies analyzed. The label (*) indicates presence of novel phylotypes (i.e. < 97% identity) and the number of phylotypes is shown inside brackets. Presence/absence data in the different sites grouped as follows: C, coastal saline ponds; I, inland haline water bodies; BS, brackish-saline waters (< 6.5% salinity); Hy, hypersaline waters (> 6.5% salinity), indicated as filled/empty squares. Discrete salinity values where the sequences were found are shown in the ellipsoid shapes at the righthand side of the plot as vertical lines within them. Bar: 0.10 fixed point mutation per nucleotide position.

and < 97% with CCM (i.e. protists that were poorly represented in culture collections but previously detected in other environmental surveys), and the 'environmental gap plot area' for protists significantly represented in cultured collections but not detected previously in environmental 18S rRNA gene surveys (i.e. < 97% with CEM and

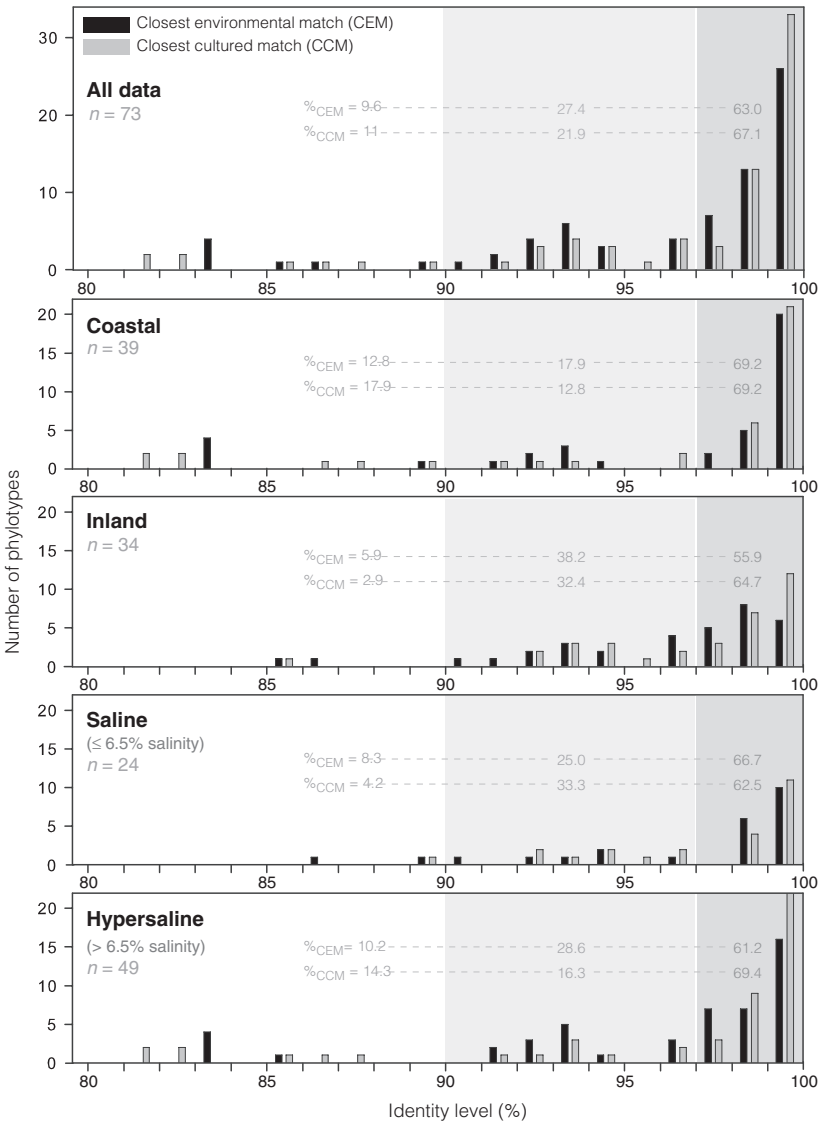

Fig. 3. Histograms showing the number of phylotypes found at different identity classes (< 90%, > 90–97%, > 97%) against the closest environmental match (CEM) and the closest cultured matches (CCM) available in databases (BLAST search, May 2012). The percentage of phylotypes fitting each identity class is indicated. Upper panel contains the whole dataset, and lower panels are separated by coastal/inland and saline/hypersaline); *n* = number of phylotypes.

Table 1. Phylogenetic diversity (PD) and phylogenetic species variability (PSV) indices calculated for different combinations of the sequence dataset

Data subset	*n*	Mean PD	SD PD	PSV	Variance
Coastal	39	3.84	0.212	0.65	< 0.001
Inland	34	5.59	0.000	0.73	< 0.001
Brackish-saline	24	4.11	0.000	0.69	< 0.001
Hypersaline	49	3.76	0.439	0.68	< 0.001
Coastal-hypersaline	27	2.06	0.330	0.63	< 0.001
Inland-hypersaline	22	2.54	0.341	0.74	< 0.001

> 97% with CCM). The remaining taxa were located on the upper righthand corner section of the plots and were considered to have limited novelty. The specific phylotypes fitting each class are shown in Table S2.

We identified the taxa allocated to the different plot sections following habitat and salinity partitioning. We observed that in coastal ponds (Fig. 4) three taxa contained 'the highest novelty', *Fungi, Cercozoa* (*Chlorarachniophyceae*) and *Choanoflagellida* (*Salpingoecidae*), whereas in inland waters up to nine taxa were considered to contain extremely novel sequences (*Bicosoecida, Choanoflagellida, Centroheliozoa, Prasinophyceae, Fungi, Telonemida, Cercozoa, Trebouxiophyceae* and *Apicomplexa*). For the 'cultured gap' we found *Spirotrichea, Prostomatea* and *Bicosoecida* for coastal ponds, and *Chlorophyceae* (*Shaeropleales*) for inland water bodies. Finally, *Heterotrichea* and *Coleochaetophycea* in coastal ponds, and *Perkinsea* and *Heterotrichea* in inland waters showed a noticeable 'environmental gap'. After salinity partitioning (Fig. 5), we observed the highest novelty within

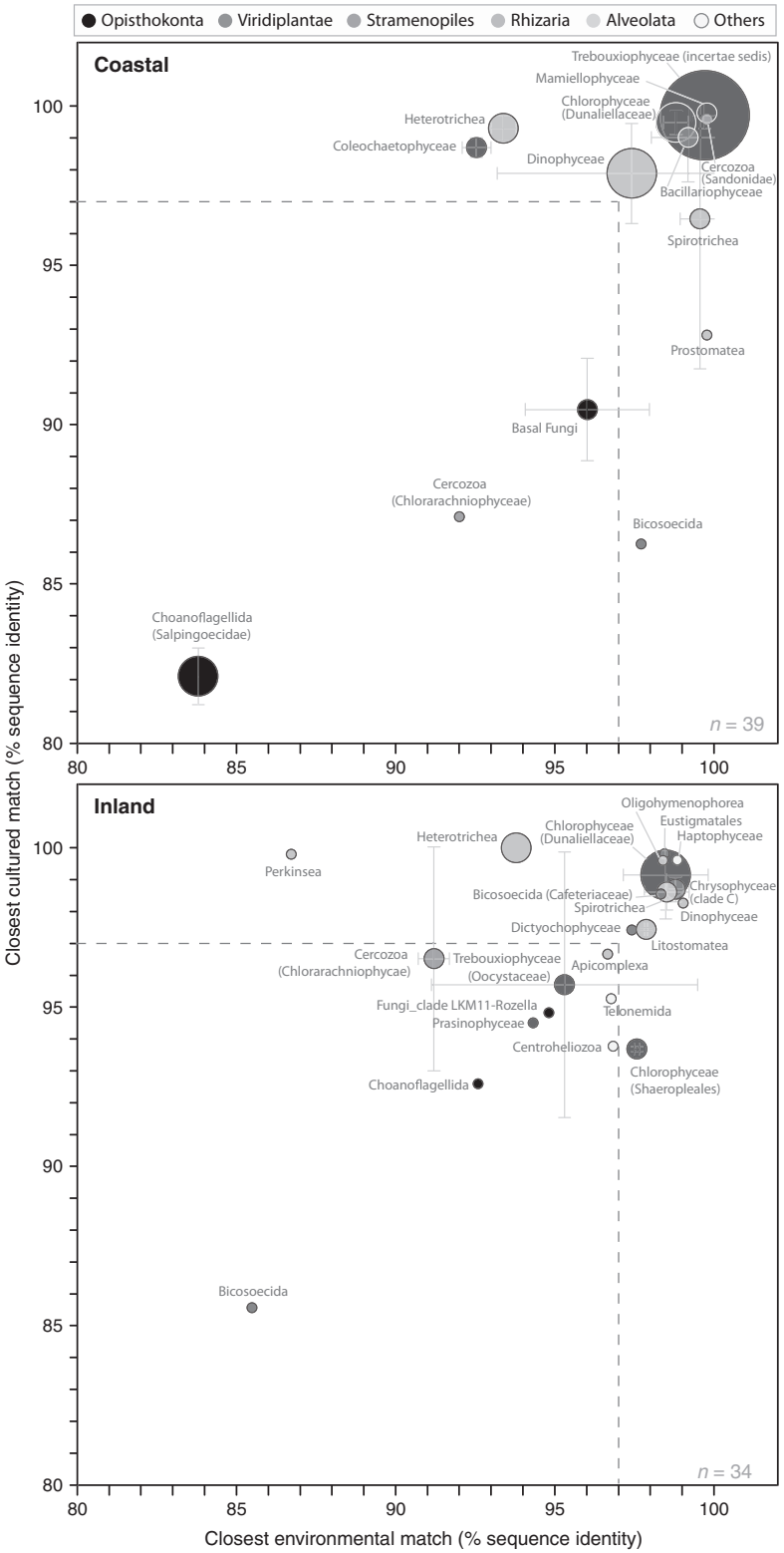

Fig. 4. Novelty pattern plot for the different eukaryal classes found in coastal saline ponds and inland haline water bodies. Closest environmental match (CEM) and the closest cultured match (CCM) available in GenBank (BLAST search, May 2012). Dots size proportional to the number of sequences; *n* = number of phylotypes.

Fungi, *Cercozoa* (*Chlorarachniophyceae*), *Prasinophyceae*, *Telonemida* and *Choanoflagellida* for the less saline ponds, and within *Choanoflagellida*, *Bicosoecida*, *Cercozoa* (*Chlorarachniophyceae*), *Centroheliozoa*, *Fungi* (*Rozella*), *Trebouxiophyceae* and *Apicomplexa* for the hypersaline. Interestingly, putative extremely novel (< 90% identity to

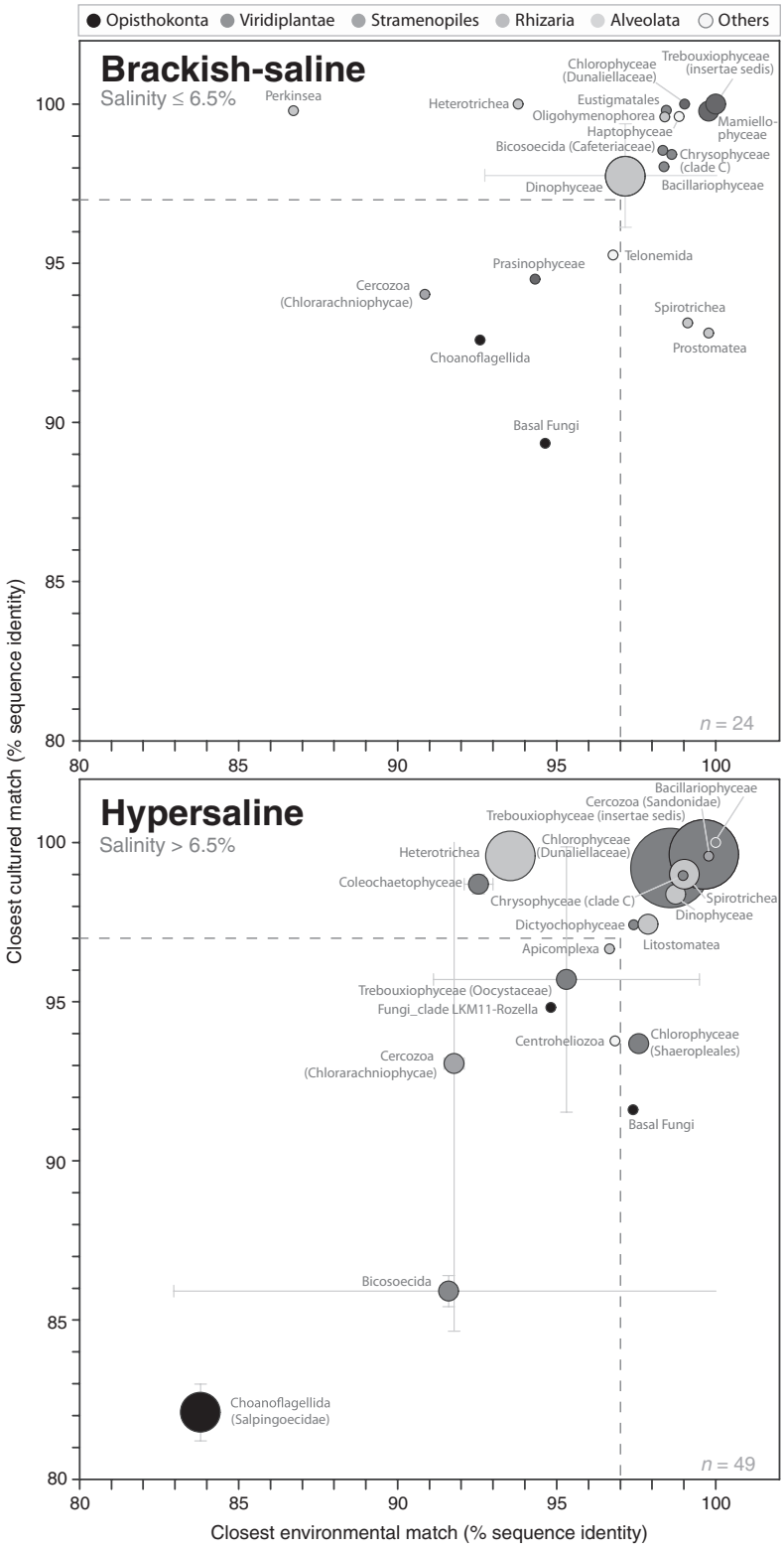

Fig. 5. Novelty pattern plot for the different eukaryal classes found in saline (< 6.5% salinity) and hypersaline (> 6.5%) water bodies. Closest environmental match (CEM) and the closest cultured match (CCM) available in GenBank (BLAST search, May 2012). Dot size is proportional to the number of sequences; *n* = number of phylotypes.

any 18S rRNA gene sequence previously reported) protists were detected both in coastal hypersaline ponds (salpingoecid-like choanoflagellates AM084310–AM084313 from several ponds in Salines La Trinitat) and in inland hypersaline ponds (bicosoecid phylotype AM179822 from Gallocanta lagoon, Table S2).

Discussion

The phylogenetic range of eukaryotic microorganisms thriving in the most extreme environments on Earth have traditionally been considered narrower than that of prokaryotes (Weber *et al.*, 2007), and mostly restricted to a limited number of groups (preferentially fungi and a few algal and heterotroph flagellated groups). In the case of high salt-adapted microorganisms it has been suggested that eukaryotes are essentially unable to cope with the selective effect of extremely high salinities (i.e. > 30% salt), and very few primary producers and protozoa have been detected under such conditions (Pedrós-Alió *et al.*, 2000; Oren, 2002). In fact, around 10 species of protozoa have been reported at salinities between 10 and 20%, whereas at extreme salinity ($\geq$ 30%) they become rare, often represented by only one or two species (Hauer & Rogerson, 2005). Although heterotrophic nanoflagellates have been shown to be active grazers in high-saline ponds of a saltern (Park *et al.*, 2003), the effect of bacterivory appears to be very limited at salinities above 25% (Guixa-Boixareu *et al.*, 1996). However, lately this limited view on protist diversity in the most extreme environments has been challenged after DNA-based environmental surveys. For instance, waters with very low pH and high concentrations of heavy metals, such as in Rio Tinto, are very rich in eukaryotes (Amaral Zettler *et al.*, 2002), and high richness has been also shown in salt crystallizers (Casamayor *et al.*, 2002; Wu *et al.*, 2009) and deep-sea brines (Alexander *et al.*, 2009; Edgcomb *et al.*, 2009), questioning the previous concept that eukaryotic life at extremely high salt concentrations is extremely limited. Interestingly, a former study in one of the Spanish multi-pond solar salterns studied here (Santa Pola, Alicante) had shown higher fingerprinting richness (OTUs) than expected along the salinity gradient, even larger or at the same level as the richness of *Bacteria* and *Archaea* (Casamayor *et al.*, 2002). In the present work, we confirmed this observation and have shown a high degree of phylogenetic novelty in salt-adapted protists. We have also shown that all of the saline ponds examined (both coastal and inland) were liable to contain a substantial novelty. However, eukaryotic assemblages thriving in inland waters require special attention, as shown by the highest value of PD for these environments. This justifies future investigations in these environments, and the development of active conservation strategies to preserve microbial biodiversity in areas often considered of minor environmental interest (Barberán & Casamayor, 2011).

The novelty level was not equally distributed among the different taxa. *Opisthokonta* and *Rhizaria* contained the most unknown organisms, whereas the *Chlorophyta* and *Alveolata* found were closer to previously reported protists. Interestingly, planktonic prasinophytes (*Viridiplantae*), an ecologically important group well represented in culture collections and marine samples (Guillou *et al.*, 2004), were also detected in saline ponds (< 6.5% salinity) but were distantly related (92% identity) to their marine counterparts (see Table S2 for percent identity to the closest match in GenBank and the original environmental source). At the most extreme conditions (> 15% salinity) phylotypes related to *Choanoflagellida, Chlorophyceae, Trebouxiophyceae, Bicosoecida, Bacillariophyceae, Cercozoa, Heterotrichea* and *Spirotrichea* were found. Their closest cultured counterparts ranged from very closely related organisms such as *Dunaliella* sp. (> 99%), *Picochlorum* sp. (> 99%), *Cylindrotheca* sp. (100%), *F. salina* (100%) and *Euplotes* sp. (98%), to very distantly related species such as *Salpingoeca* sp. (81–83% identity) and *Cafeteria* (86%). Overall, we highlighted important gaps existing in both culturing and sequencing efforts for non-marine (hyper)saline water bodies, revealing interesting novel phylotypes to be investigated in more detail in the future, but also phylotypes with a potential cosmopolitan distribution. Examples of closely related phylotypes (i.e. identity > 98.5%) were detected in Perkinsea found in a doline in Llamará (Chile) and as a cultured parasite from a Catalan harbor (EU502912), or in *F. salina* observed in the different water bodies of this study and other water bodies in Italy and Australia, and *Dunaliella* sp. studied in Australia, the Arctic Ocean, and Indian coastal waters. Interestingly, a sequence related to a bicosoecid (AM084289) detected in a hypersaline pond of Santa Pola solar salterns was closely related (> 98.5%) to an unknown picoeukaryote in a hypersaline lake in the Eastern Tibet Plateau (Wu *et al.*, 2009) but was distantly related (< 87%) to any cultured protist. This is an example of a widely distributed extremely novel protist that deserves to be brought into culture, although whether these are true cosmopolitans remains to be investigated. The sequences displaying the most extreme novelty were all recovered from hypersaline waters, and they were phylogenetic placed related to other marine species, although in a distinctive clade. However, proper phylogenetic placement will require longer 18S rRNA gene sequences after full gene cloning. A recent work has identified bicosoecid cohorts for both moderately and extreme hypersaline environments (Park & Simpson, 2010) and this would probably apply for other protist taxa considered today to be properly characterized, such as the novel phylotypes found for *Choanoflagellida* (AM084310–AM084313) related to choanoflagellate clade A, and *Bicosoecida* (AM179822) related to the marine bicosoecids *Cafeteria* (Del Campo & Massana, 2011).

The degree of novelty in the dataset was obtained after averaging the identity values for all sequences within a given taxon (Del Campo & Massana, 2011). Extreme care

was taken with the analysis of the most divergent sequences, to rule out the presence of pseudogenes (Thornhill *et al.*, 2007; Massana *et al.*, 2011) in the dataset. On the one hand, we analyzed the secondary structure of the gene fragments using ARB, which was then compared with the structure obtained from the 18S rRNA gene of cultured strains. On the other hand, our dataset focused on the most abundant bands excised from the DGGE gel (an indication that we were dealing with abundant products in the PCR mixture), and matched several sequences previously reported in databases (e.g. salpingoecid-like *Choanoflagellida*). Curiously, the mean identity values recorded for both CEM and CCM were rather similar, and in a few cases we found CCM > CEM identities. This is an unusual finding after more than a decade of environmental DNA surveys in eukaryotes. For instance, in marine surveys CEM > CCM identities are usually reported (Massana *et al.*, 2011), showing that inland and coastal saline water bodies have attracted more attention from traditional protist microbiologists than microbial (eukaryal) ecologists. These environments emerge as important reservoirs of largely unseen microbial eukaryotic biodiversity with a phylogenetic richness and novelty far greater than previously suspected.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Sites studied and several key environmental parameters measured.

Table S2. The closest environmental match (CEM) and the closest cultured match (CCM) in GenBank after a BLAST search (May 2012) for the different 18S rRNA gene sequences obtained in this study with detailed accession numbers.

Table S3. Pairwise comparisons for the phylotypes found in each major eukaryal group and environment of origin.