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Assessment of yeast diversity in a marine environment in the south of portugal by microsatellite-primed PCR

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Abstract

The occurrence and diversity of yeasts in seawater was investigated in a study site located 20 Km off Faro, Portugal, above the Álvares Cabral Trench. A total of 43 water samples from different layers (above the permanent thermocline, under the thermocline and near the bottom) and directly from the surface, originated 234 isolates. All the isolates were identified using a molecular approach that included, in a first stage, MSP-PCR fingerprinting. A total of 31 MSP-PCR classes were formed, 8 for the pigmented yeasts and 23 for the non-pigmented yeasts. The pink coloured isolates were identified by direct comparison of the new fingerprints with those obtained for representative strains of the various species. For identification of the non-pigmented yeasts, a representative isolate of each MSP-PCR class was selected for sequence analysis and compared with reference sequences. The five most abundant yeast species were *Sakaguchia dacryoidea, Pseudozyma aphidis, Rhodosporidium babjevae, R. diobovatum* and *Debaryomyces hansenii*. The distribution of isolates and species in the major taxonomic groups indicated that the number of basidiomycetous yeasts and their diversity are prevalent in relation to their ascomycetous counterpart. Diversity indices were determined and superficial water and water near the bottom had the highest diversity. The sampling effort effectiveness was estimated, and found to correspond to approximately 60% of the species present. MSP-PCR identification proved suitable for pigmented basidiomycetous yeasts and, when used in conjunction with sequence analysis, was effective for the characterization of non-pigmented populations. Our results indicate that the MSP-PCR fingerprinting method is appropriate for the characterization of large groups of isolates due to its simplicity and good reproducibility.

Introduction

Yeasts are distributed among several phylogenetic groups of fungi and are classified in two phyla, Ascomycota and Basidiomycota. Macroscopically, the yeasts can be divided in two groups based on colony pigmentation. One group includes the species that produce pink, salmon or reddish colonies and, with the exception of a few cases, the vast majority belong to the Basidiomycota. The other group includes species forming white or cream-coloured colonies, and its members are classified both in the Ascomycota and in the Basidiomycota.

It has long been known that yeasts occur in aquatic habitats, both in freshwater environments (Spencer et al. 1964; van Uden and Ahearn 1963) and in marine environments (Fischer and Brebeck 1894; van Uden and Fell 1968). Using conventional microbiological techniques, it was previously observed that yeasts are the dominant fungi in oceans (Sieburth 1979). In marine waters, yeast populations normally decrease with increased depth and increased distance from land. However, plankton blooms, surface slicks, current boundaries, eddies and thermoclines may alter this pattern and yeast cell densities may rise above 10^3 per litre. The number of viable yeast cells per litre was found to vary between 13 (North Pacific off Japan) and 274 (off La Jolla, California) (van Uden and Fell

1968). Due to discontinuities found in the distribution of yeasts in sea water, a microzonation scenario, eventually correlated with analogous discontinuities found in the distribution of utilizable organic matter, was suggested (Kriss 1959; Kriss and Novozhilova 1954; Kriss et al. 1952). Moreover, it is well known that many yeast species reported to occur in marine waters are also recovered from terrestrial environments.

Quantitative studies on the occurrence and distribution of yeasts in marine environments were abandoned after 1970 therefore the characterization of their ecological roles and the elucidation of the microzonation phenomena have advanced since then. Presently, several methods that avoid the cultivation step have been proposed for the characterization of natural microbial populations. The advantages of such approaches are immense but contradictory results were obtained when the conventional isolation procedures were carried out in parallel with the direct detection method (Yang et al. 2001).

When culture-dependent approaches are employed, environmental samples normally yield a large number of isolates. Moreover, their identification by physiological and morphological criteria is time consuming and, in many cases, inconclusive. Modern approaches to yeast identification include sequence analysis of selected regions of DNA, namely the D1/D2 domain of the 26S rDNA, a fragment of approximately 600–650 bp. Normally, strains of the same species have identical D1/D2 sequences or no more than two mismatches (Kurtzman and Robnett 1998; Fell et al. 2001). However, in spite of its accuracy, this method is impracticable as a first approach for the characterization of large numbers of isolates. In order to overcome such problems, usually only a fraction of the total number of each type of colony is selected for identification down to the species level. It is assumed that all the remaining similar colonies belong to the same species. However, this approach has flaws that can affect the overall result of the experiment since it is well known that different species can have indistinguishable colonies, or that the same species can have more than one colony type.

We have developed a quick and reliable protocol using molecular approaches for the accurate identification of all the yeasts isolated in culture. The micro/minisatellite-primed PCR (MSP-PCR) method was used gives good results obtained in the differentiation and identification of several basidiomycetous red yeasts (Gadanho et al. 2001; Sampaio et al. 2001a, 2001b). This PCR fingerprinting method employs a single micro/minisatellite primer in the PCR reaction and, in general, conserved profiles are obtained for strains belonging to the same species (Gadanho and Sampaio 2002). Other authors have also successfully applied this method, namely for the epidemiological characterization and identification of pathogenic yeasts such as *Cryptococcus neoformans* (Meyer et al. 1993) and *Candida* spp. (Meyer et al. 2001) and for the characterization of mycorrhizal fungi (Longato and Bonfante 1997).

This approach was used in the present study to investigate the occurrence and diversity of yeasts in the water column at a study site located 20 km off Faro in the South of Portugal, above the head of the Álvares Cabral Trench. All yeast isolates were characterized and identified by a molecular approach that included, in a first stage, fingerprinting using MSP-PCR and, in selected cases, the sequence analysis of the D1/D2 region of the 26S rDNA.

Materials and methods

Sampling

Sampling was performed during cruise SIRIA2000-1 on board the NRP ''Auriga'' in January 2000. The study site was located about 20 Km South of Faro, Portugal, above the Álvares Cabral trench head. Samples were taken at four different stations as shown in Figure 1. Water samples were collected with Niskin bottles at various depths: immediately above the permanent thermocline (80–100 m), under the thermocline (200–500 m) and near the bottom (−700 m), and directly from the surface. Forty-three samples of 1000 ml were taken during a 24 h period, corresponding to a full tidal cycle.

Yeast isolation

Each sample was divided in two portions of 500 ml that were immediately filtered using 0.45 μm pore size filters of 47 mm diameter, which were placed on MYP agar [0.7% (w/v) malt extract, 0.05% (w/v) yeast extract, 0.25% (w/v) soytone and 1.5% (w/v) agar] supplemented with 0.05% (w/v) chloramphenicol. In order to monitor the relevance of contamination during the sample manipulation and the filtering process, air was filtered using the same procedure, during a similar period of time. The inocu-

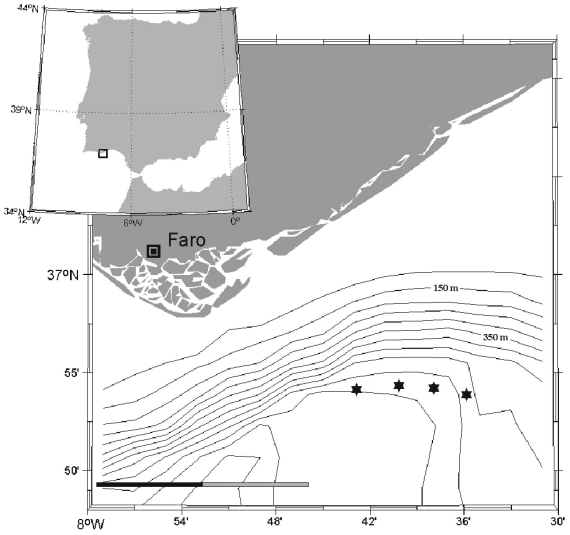

Figure 1. Locations of the four sampling sites (☆). Scale bar = 20 Km.

lated plates were incubated at 14 °C for 5–7 d. Transfers were made for all colonies obtained. Purified cultures were stored at 4 °C after microscopic examinations were made to ensure that no bacterial isolates had been selected.

DNA extraction

An existing protocol (Sampaio et al. 2001a) was employed with some modifications in order to increase the rapidity of the procedure. Two loopfuls of MYP agar grown cultures were suspended in 500 μl lysing buffer (50 mmol l^{-1} Tris, 250 mmol l^{-1} NaCl, 50 mmol l^{-1} EDTA, 0.3% w/v SDS, pH 8) and the equivalent to a volume of 200 μl of 425–600 μm glass beads (Sigma) was added. After vortexing for 3 min, the tubes were incubated for 1 h at 65 °C. The suspensions were then centrifuged for 10 min. Finally, the collected supernatant was diluted 1:750 and 5 μl were directly used in the PCR. The remaining supernatant was immediately conserved at −20 °C.

MSP-PCR fingerprinting

The microsatellite primers $(GTG)_5$ and $(GAC)_5$ were used as previously described (Gadanho and Sampaio 2002). All PCR reactions were performed in 25 μl reaction volumes containing 1 X PCR buffer (Pharmacia, Biotech), 2 mmol l^{-1} of each of the four dNTPs (Promega), 0.8 μmol l^{-1} of primer, 5 μl of the diluted supernatant containing the genomic DNA and 1U of *Taq* DNA polymerase (Pharmacia, Biotech). Amplification was performed in a Uno II Thermal Cycler (Biometra), consisting of an initial denaturation step at 95 °C for 5 min, followed by 40 cycles of 45 s at 93 °C, 60 s at 50 °C and 60 s at 72 °C and a final extension step of 6 min at 72 °C. A negative control in which DNA was replaced by sterile distilled water was also included. Amplified DNA fragments were separated by electrophoresis in 1.4% (w/v) agarose gel (GIBCO, BRL), in 0.5 X TBE (Tris-Borate-EDTA) buffer at 90 V for 3.5 h and stained with ethidium bromide. On each gel, a molecular size marker was used for reference (λ DNA cleaved with *Hind*III and ΦX174 DNA cleaved with *Hae*III - Pharmacia, Biotech).

DNA banding patterns were visualized under u.v. transillumination and images were acquired using a Kodak Digital Science EDA 120 System and the Kodak Digital Science 1D Image Analysis Software. All fingerprints obtained were grouped by similarity using Gel Compar 4.1 (Applied Maths 1998) and the Pearson correlation coefficient. A visual confirmation of each group was performed and minor adjustments were made. Finally each group was assigned in a distinct MSP-PCR class. Identification of the pink coloured yeasts was performed using the identification module and the Pearson correlation coefficient with the same software.

rDNA sequence analysis

Total DNA was extracted using the method described above and amplified using rDNA primers ITS5 (5′GGA AGT AAA AGT CGT AAC AAG G) and LR6 (5′CGC CAG TTC TGC TTA CC). Cycle sequencing of the 600–650 base pair region D1/D2 at the 5′ end of the 26S rDNA domain employed forward primer NL1 (5′GCA TAT CAA TAA GCG GAG GAA AAG) and reverse primer NL4 (5′TCC TCC GCT TAT TGA TAT GC). Sequences were obtained with an Amersham Pharmacia ALF express II automated sequencer using standard protocols. Sequences obtained in this study have accession numbers from AF485963 to AF486000 and a detailed list is available at http://www.crem.fct.unl.pt/dimorphic_basidiomycetes. Phylogenetic trees and additional information concerning the isolates are also available at this web site. For identification, the

obtained sequences were compared with those of all known yeast species, available at the GenBank database.

Diversity analyses

Simpson's index (λ) and Shannon's index (H′) were calculated (Ludwig and Reynolds 1988). The first one gives the probability that two individuals drawn at random from a population belong to the same species. Therefore the lower the index, the higher the diversity. The Shannon's index measures the degree of uncertainty in predicting to what species an isolate chosen at random from a community will belong. Therefore, the higher the index, the higher the diversity and the evenness. These two indices were also used to calculate Hill's numbers of diversity, N1 and N2. They represent the number of abundant species and the number of very abundant species, respectively (Ludwig and Reynolds 1988). As a measure of evenness we calculated the modified Hill's ratio E5, which approaches zero as a single species is dominant in a community (Ludwig and Reynolds 1988). All these indices were used to compare the yeast diversity (species richness and evenness) in the four water layers.

Computation of accumulation statistics was performed with EstimateS 6.0b1 software (Colwell 1997). A combination of parametric and non-parametric richness estimators was used. To access the expected species diversity we employed the Abundance-Based Coverage (ACE) and the Incidence-Based Coverage (ICE) estimators (Chao et al. 1993; Chazdon et al. 1998; Lee and Chao 1994). The sampling effort effectiveness was evaluated through a rarefaction plot of Coleman estimator (Coleman 1981) and observed species. Calculations were performed using a pooling method designed to evaluate the accuracy of the estimator value (Colwell and Coddington 1994).

Results

Yeast isolation

A total of 43 water samples of 1000 ml were analysed and in 30 of them yeast isolates were obtained (Figure 2). Purification of all colonies was performed between the fifth and seventh day after filtration of the water samples. This incubation period was set in order to obtain a satisfactory growth of the yeast colonies but to avoid overgrowth by filamentous fungi. The number of colony forming units obtained from each positive sample ranged between 1 and 66. In total, 234 yeast isolates were obtained and their distribution across the water column is shown in Figure 2. The complete list of isolates and information related to them is available at http://www.crem.fct.unl.pt/dimorphic_basidiomycetes. Seven of the thirteen negative samples corresponded to water samples collected above the thermocline but, nevertheless, this type of water yielded the largest number of isolates (Figure 2). Bacteria resistant to 0.05% chloramphenicol were found only in one sample from which no yeasts were detected.

In order to proceed to MSP-PCR fingerprinting, all the yeasts were grouped based on a few macromorphological features like colony colour and texture (mucous or butyrous). About 60% of the yeasts were pink coloured (141 isolates). Among the 93 non-pigmented isolates, 44% (41 isolates) presented identical cream coloured colonies with margins becoming darker after a few days.

MSP-PCR fingerprinting

In order to access the reproducibility of the simplified DNA extraction protocol, several dilutions from 1:100 to 1:1500, of the crude DNA extract were

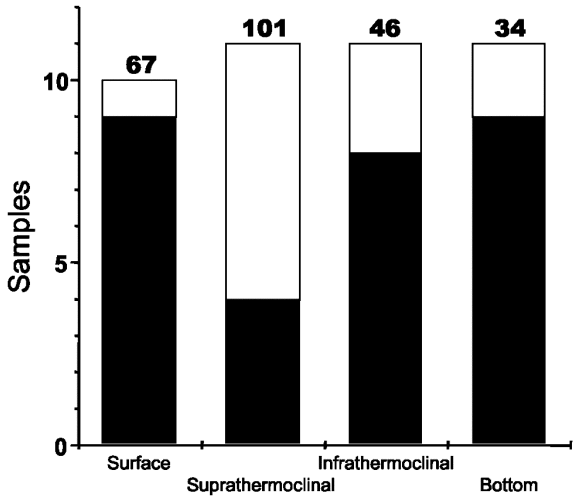

Figure 2. Number of samples collected from the four water layers. Each black bar indicates the number of positive samples (those yielding yeasts) and negative samples are shown in white. Numerals above each bar indicate the number of isolates recovered from each water layer.

performed. No differences were detected in the MSP-PCR fingerprints obtained, except in the case of very faint amplicons. Fingerprints obtained after storage for 18 months at −20 °C of the crude DNA, did not differ from the original ones. Furthermore, DNA from selected isolates was extracted using both the simplified method and the complete protocol (Sampaio et al. 2001a) and identical results were obtained. In the MSP-PCR study with primer $(GTG)_5$ all the isolates were investigated and the obtained fingerprints were grouped by global similarity. The minor variability between the fingerprints of a given MSP-PCR class was regarded as intraspecific heterogeneity or possible variability caused by the method. A total of 31 classes were established, 8 for the pigmented yeasts and 23 for the non-pigmented yeasts. The majority of the isolates (80%) were grouped in four classes. The remaining yeasts were distributed in 27 classes, 16 of which were composed by a unique isolate. The representative MSP-PCR classes are shown in Figures 3 and 4 for the pigmented and non-pigmented yeasts, respectively.

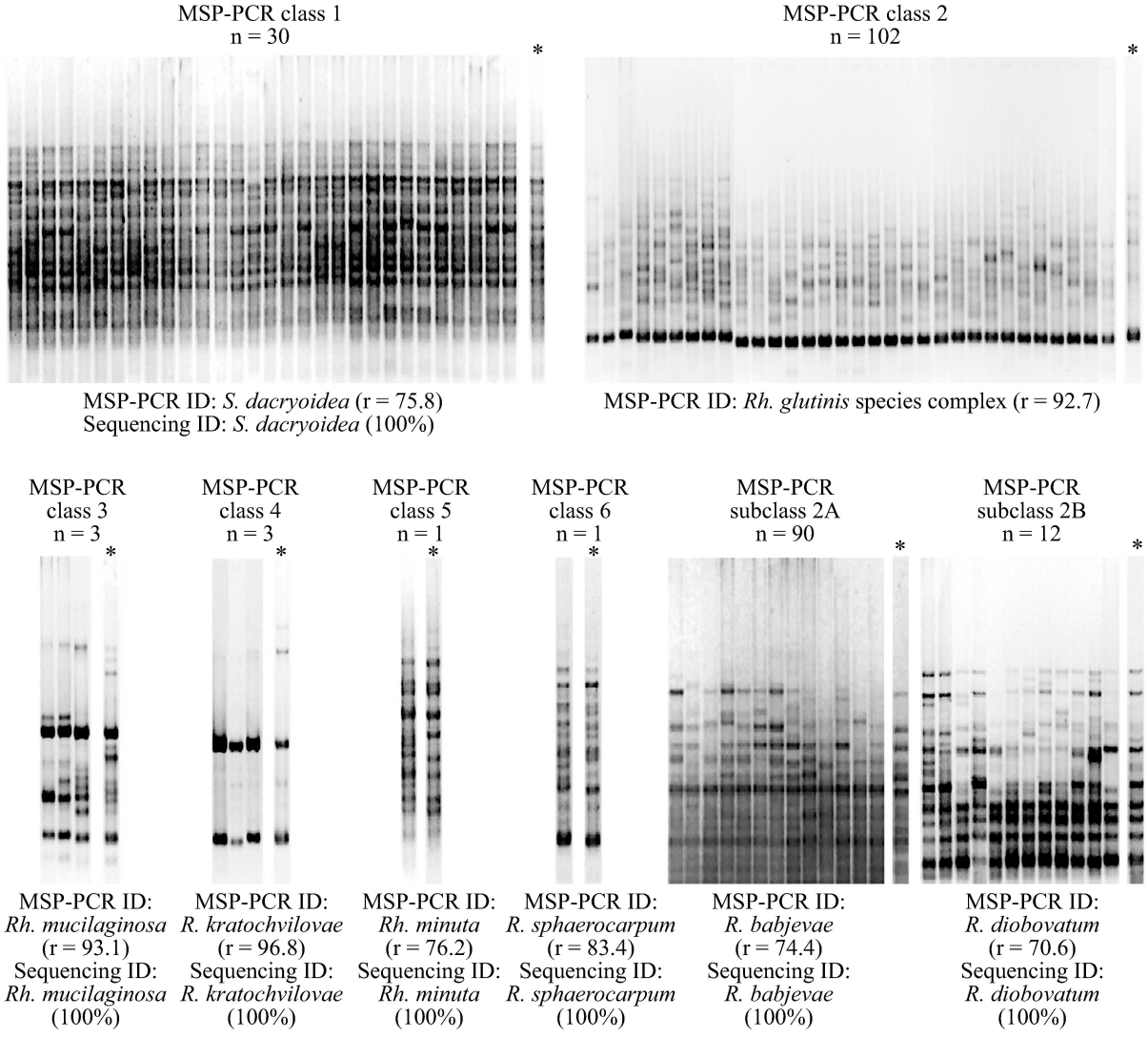

Figure 3. MSP-PCR classes obtained with primers $(GTG)_5$ (classes 1 to 6) and $(GAC)_5$ (sub-classes 2A and 2B) for pigmented yeasts. Results of MSP-PCR identification (MSP-PCR ID) and 26S rDNA sequencing (sequencing ID) are also shown. For classes 2 and 2A only a fraction of the profiles is shown. n – number of isolates; r – average Pearson correlation coefficient obtained between the profiles of all the isolates of each class and reference strains of the closest species; percent values indicate sequence homology; * - MSP-PCR profile of the type strain of the closest species of each class. For abbreviations of species names see legend of Figure 5.

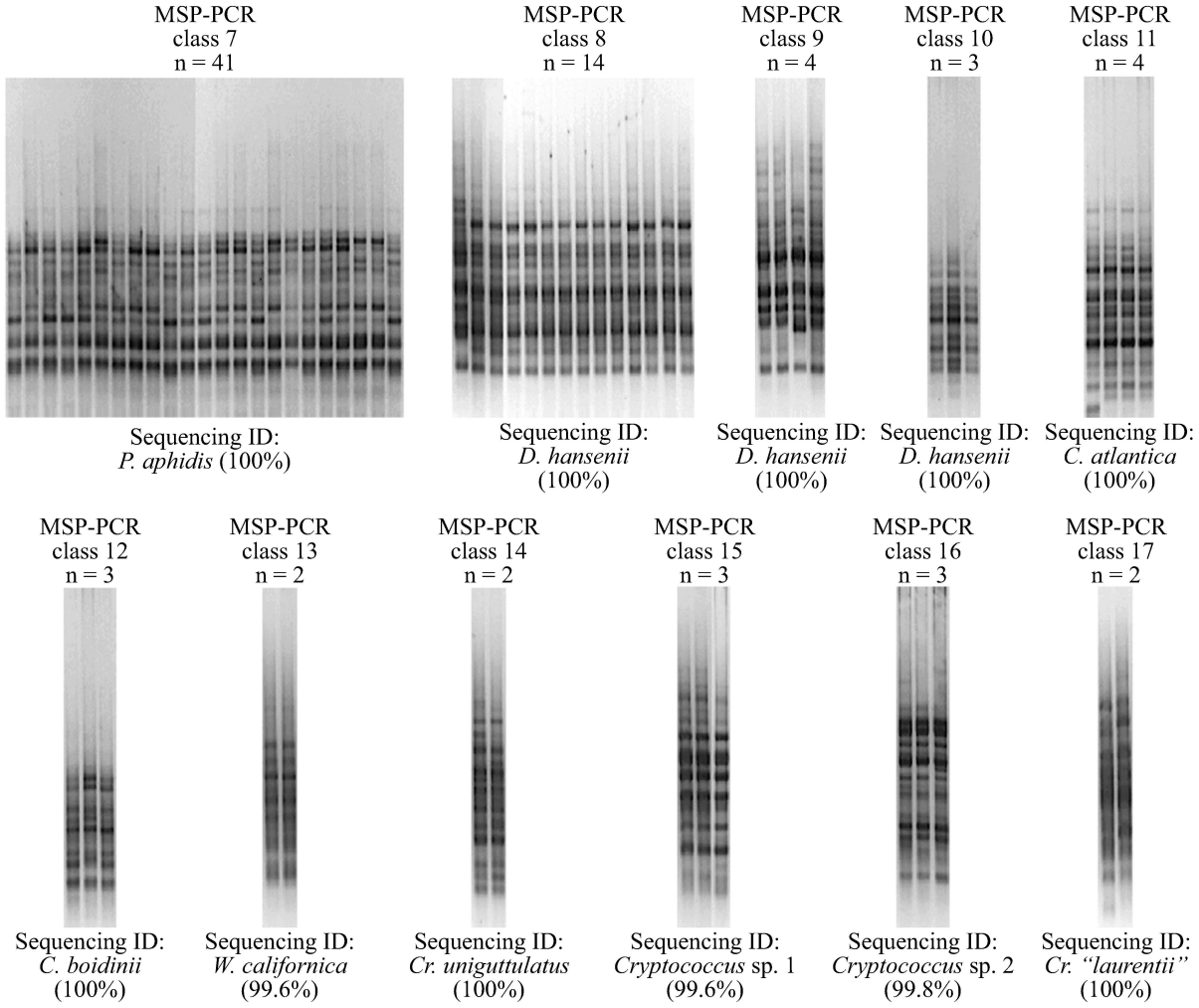

Figure 4. MSP-PCR classes obtained with primer $(GTG)_5$ for the non-pigmented yeasts. Classes with a single isolate are not shown. Results of 26S rDNA sequencing (sequencing ID) are also shown. For class 7 only a fraction of the profiles is shown. n – number of isolates; percent values indicate sequence homology. For abbreviations of species names see legend of Figure 5.

Species identification

In order to identify all the isolates two strategies were employed. In the case of the pink coloured yeasts the identification was based on the comparison with reference MSP-PCR fingerprints available in a database that we have been compiling at our laboratory for several years, as shown in Figure 3. For the non-pigmented yeasts, since no such database was available, the strains were first sorted according to their MSP-PCR fingerprints and one or more representatives of each group was sequenced. These sequences were then compared with reference sequences available at the GenBank database. The results are shown in Figure 4.

Only two of the 141 pink isolates could not be identified with the MSP-PCR approach (fingerprints not shown). Sequence analysis of the D1/D2 region of the 26S rDNA indicated that one of the isolates had a sequence identical to *Rhodotorula nothofagi* and the other had two mismatches to the type strain of *Sporobolomyces roseus*. The $(GTG)_5$ fingerprints obtained for class 2, corresponded to the *Rhodotorula glutinis* species-complex. For species discrimination within this complex, the isolates were investigated with a second MSP-PCR primer according to Gadanho and Sampaio (2002). When primer $(GAC)_5$ was used, two sub-classes corresponding to *Rhodosporidium babjevae* (sub-class 2A) and to *R. diobovatum* (sub-class 2B) were obtained (Figure 3). In order to confirm the MSP-PCR identification results for the pigmented isolates, a representative strain

of each MSP-PCR class was investigated by sequence analysis. The sequence data validated the MSP-PCR identification as shown in Figure 3.

A rank-abundance/incidence plot of all yeast species collected is presented in Figure 5. The identity of the five most abundant yeast species (more than 10 isolates each) was determined with a high degree of confidence since the sequences obtained in the present study matched exactly the reference sequences deposited at the GenBank database. *Rhodosporidium babjevae*, in spite of being the most abundant species (90 isolates) was isolated only from five samples, whereas *Pseudozyma aphidis*, the second most abundant (41 isolates) was found in 17 samples (Figure 5).

Discussion

A higher number of MSP-PCR classes, was found among the non-pigmented yeasts (23 classes) by comparison with the pink coloured yeasts (8 classes, one of which further divided into two sub-classes). The MSP-PCR class 1 (Figure 3) corresponded to *Sakaguchia dacryoidea*, a species originally found in the Antarctic Ocean at depths ranging from 3 to 3900 m (Fell et al. 1973). In our study, 93% of the *S. dacryoidea* isolates were found in superficial water. This yeast is rarely collected and there are no reports of its occurrence on terrestrial or freshwater environments. Among the various yeast species detected, *S. dacryoidea* is the best candidate for an autochthonous marine yeast since it is only found in seawater, it is one of the most abundant species found in this study and it was detected in a considerable number of samples (Figure 5).

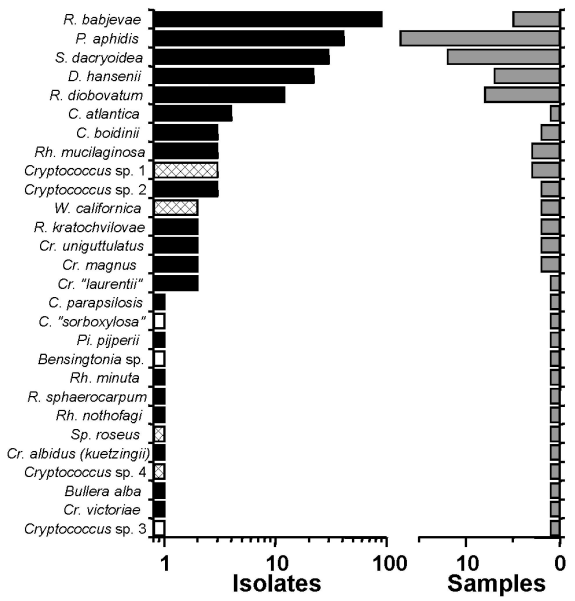

Figure 5. Rank-abundance (left side) and rank-incidence (right side) plot for all the yeast species collected. On the abundance plot, black bars indicate identification with high level of confidence (100–99.8% sequence homology corresponding to 0–1 discrepant nucleotides); squared bars indicate identification with intermediate level of confidence (99.7–99.6% sequence homology, 2 discrepant nucleotides); and open bars indicate identification with low level of confidence (99.3–98.8% sequence homology, 3–7 discrepant nucleotides). Abbreviations: *C.*, *Candida*; *Cr.*, *Cryptococcus*; *D.*, *Debaryomyces*; *P.*, *Pseudozyma*; *Pi.*, *Pichia*; *R.*, *Rhodosporidium*; *Rh.*, *Rhodotorula*; *S.*, *Sakaguchia*; *Sp.*, *Sporobolomyces*; *W.*, *Williopsis*.

The MSP-PCR class 2 corresponded to the *Rhodotorula glutinis* species-complex, which includes another three taxa, *Rh. graminis, Rhodosporidium babjevae* and *R. diobovatum* (Gadanho and Sampaio 2002). Because they are very closely related, the four species are presently difficult to distinguish, even by molecular methods. Additional MSP-PCR experiments made with primer $(GAC)_5$ indicated that the majority of our isolates (90 strains) belonged to *R. babjevae* (sub-class 2A) and 12 strains were identified as *R. diobovatum* (sub-class 2B). *Rhodosporidium diobovatum* is frequently isolated from seawater (Newell and Hunter 1970; Nagahama et al. 2001) and we found this species in the four water layers investigated. In this study, the incidence of *R. diobovatum* was considerable (Figure 5). *Rhodosporidium babjevae* seems to be associated with terrestrial habitats (Golubev 1993). These results question the presumed ecological specialization of *R. babjevae*. However, since the correct identification of the species of the *Rh. glutinis* complex has been problematic, the undetected presence of *R. babjevae* in marine samples might have occurred in the past. Among the 90 isolates of *R. babjevae*, 88 were collected in three consecutive samples taken near the bottom and above and under the thermocline. Considering the five most abundant species, *R. babjevae* showed the lowest incidence. This distribution is, at present, difficult to interpret. A microzonation pattern can be tentatively advanced. In this scenario, the occasional availability of organic matter would allow a dramatic increase in *R. babjevae* populations. According to this hypothesis, our large collection of *R. babjevae* isolates is more the result of a chance-dependent event than the manifestation of an ecological trend.

Regarding the non-pigmented yeasts, class 7 corresponded to *Pseudozyma aphidis* (Figure 4). This species showed the highest incidence and was the

second most abundant with respect to the number of isolates collected (Figure 5). *Pseudozyma aphidis* was originally isolated from secretions of aphids on leaves of *Solanum pseudocapsicum* (Henninger and Windisch 1975). However, the occurrence of yeasts of the genus *Pseudozyma* in giant white clams of the genus *Calyptogena* collected in the Northwest region of the Pacific Ocean was recently reported (Nagahama et al. 2001). *Pseudozyma aphidis* was isolated from the various water layers sampled and the relative distribution of the 41 isolates was as follows: 27% in superficial water; 49% in suprathermoclinal water; 12% in infrathermoclinal water and 12% in water near the bottom.

Four MSP-PCR classes (classes 8, 9, 10 and 18) corresponded to *Debaryomyces hansenii* var. *hansenii* based on the analysis of the D1/D2 region of the 26S rDNA. This species is consistently found in seawater (Hagler and Ahearn 1987) and, overall, we collected 22 isolates. In our seawater samples this species was the fourth most frequent and the most abundant ascomycetous yeast (Figure 5). Microscopically, all the isolates selected for sequence analysis formed single, round and warty ascospores, typical for this species. In this case, the MSP-PCR profiles were not conserved among the isolates. It is possible that the different subgroups represent different populations within the species or, eventually, that they reflect taxonomic heterogeneity. In three of the seven water samples that yielded *D. hansenii* isolates, two distinct MSP-PCR profiles were found.

Among the yeast taxa with few representatives (less than five isolates), we detected six ascomycetous species and 16 basidiomycetous species. Within the ascomycetes, the isolation of four cultures of *Candida atlantica* is relevant since this species is known only from one strain isolated from shrimp eggs in the North Atlantic Ocean. Our isolates of *Williopsis californica* formed ascospores similar to those of the reference strains but the sequence determined in our study differs in two bases from the sequence of the type strain of the species. There are no records of isolation of *W. californica* from marine waters although one strain deposited at the CBS collection was isolated from shrimp (Kurtzman 1998a). *Pichia pijperii, Candida parapsilosis* and *C. sorboxylosa*, have not been found in aquatic environments, whereas *C. boidinii* has a widespread occurrence, which includes seawater (Kurtzman 1998b; Meyer et al. 1998).

Among the poorly represented basidiomycetous pigmented yeasts, we found *Rhodosporidium sphaerocarpum*, *Rhodotorula mucilaginosa* (synonym *Rh. rubra*) and *Rh. minuta*, which have also been isolated in other surveys (Hagler and Ahearn 1987). With respect to the non-pigmented yeasts, *Cryptococcus laurentii* has been reported to occur in seawater (Hagler and Ahearn 1987). Presently, the taxonomic status of this species is confusing since phenotypic (nutritional) and molecular (sequence data) circumscriptions do not coincide. Since none of our isolates originated a D1/D2 rDNA sequence identical to the one of the type strain of this species, we avoided the use the specific epithet *laurentii* to designate them. *Cryptococcus* sp. 2 and *Cryptococcus* sp. 3 have sequences similar to the one of CBS 9007, labelled *Cryptococcus* aff. *laurentii*. In the past, nutritional identifications probably would have included both unidentified species in *Cr. laurentii*. It is also possible that several isolates identified in the present study as *Cr. albidus* var. *kuetzingii* and *Cr. magnus* would have been included in *Cr. albidus* based on physiological profiles. According to our molecular identifications, six of the 28 species found (21%), might represent undescribed taxa.

The isolation of ballistoconidia-producing yeasts like *Bullera alba* and *Sporobolomyes roseus*, typical inhabitants of the phyllosphere, confirms that at least a fraction of the sampled organisms may not represent autochthonous yeast populations. Both species were represented by one isolate each and were found in water samples taken above the thermocline. The detection of such yeasts off the continental shelf at considerable depths suggests that they are capable of maintaining viability during prolonged periods and at environmental conditions that differ substantially from those of their original habitat. Yeasts of the genus *Sporobolomyces* were previously detected in sea water 45 miles off the West Coast of Baja California, Mexico (Hernandez-Saavedra et al. 1992). Phyllosphere yeasts of the genus *Sporidiobolus* and *Sporobolomyces* were found in benthic organisms (mussels and clams) collected in deep-sea environments in the Northwest Pacific Ocean (Nagahama et al. 2001).

The diversity indices calculated for the water layers studied were the Simpson's index, the Shannon's index and the Hill's numbers (Table 1). They indicated that the superficial water and the water near the bottom had the highest diversity. In spite of yielding the largest number of isolates, the suprathermoclinal water showed the lowest diversity, as revealed by Hill's numbers of abundant and very abundant species

(N1 = 3.22 and N2 = 2.33), Shannon's index (H′ = 1.17) and Simpson's index (λ = 0.429). In order to measure the species diversity two parameters are relevant, the species richness (total number of species isolated) and the evenness (distribution of isolates among species). Taking into account the number of isolates, the water near the bottom had a considerable species richness (N0 = 12, n = 32), when compared to the superficial water (N0 = 19, n = 66). Furthermore, the water near the bottom presented the highest evenness (E5 = 0.935) in contrast with the lowest evenness of the superficial water (E5 = 0.496). This combination of species richness and evenness shows that superficial and bottom water have a similar yeast diversity, which is higher than the diversity found in the two other water layers studied.

In this study, yeast diversity evaluation seems to correspond to approximately 60% of the species present in the water column. This value was obtained by comparing the accumulation curves for observed species with the Coleman estimator and the ACE/ICE final values for the global data set (Figure 6). There were sensible differences among the subsets corresponding to the various water layers (data not shown). The highest ratio was observed for the infrathermoclinal layer where approximately 56% of the yeast community was found. Bottom (48%) and suprathermoclinal (47%) layers followed. In the plot, the vertical comparison of the distance between Coleman estimator and species accumulation (Colwell and Coddington 1994) indicated that the suprathermoclinal layer had the highest patchiness (heterogeneity). The surface layer (< 36%) stands apart because ACE/ICE estimators did not yield conclusive results. Considering the pattern of yeast distribution in seawater, the sampling effort employed in the present study for the assessment of yeast diversity seems to have been reasonably adequate. To obtain a better species coverage, a considerable higher sampling effort had to be made.

Table 1. Diversity values obtained for each water layer.

	S	ST	IT	B
H′	2.09	1.17	1.40	1.95
λ	0.222	0.429	0.395	0.151
N0	19	8	9	12
N1	8.08	3.22	4.06	7.03
N2	4.51	2.33	2.53	6.64
E5	0.496	0.599	0.500	0.935

S – surface, ST – suprathermoclinal, IT – infrathermoclinal, B – bottom, H′ – Shannon's index, λ - Simpson's index, N0 – total number of species obtained (species richness), N1 – Hill's number of abundant species, N2 – Hill's number of very abundant species, E5 – evenness modified Hill's ratio.

The distribution of isolates and species in the major taxonomic groups indicated that the number of basidiomycetous isolates (200 isolates) and their diversity (21 species) are prevalent in relation to their ascomycetous counterpart (34 isolates, 7 species). Species of the three classes that form the phylum Basidiomycota were collected. The classes Urediniomycetes (*Rhodotorula* and related genera) and Hymenomycetes (*Cryptococcus* and related genera) were represented by 10 species each and the Ustilaginomycetes by only one. With respect to the number of isolates, the hymenomycetous yeasts were found in the lowest levels (17 isolates), the Ustilaginomycetes had 41 isolates and the dominant group corresponded to the urediniomycetous yeasts

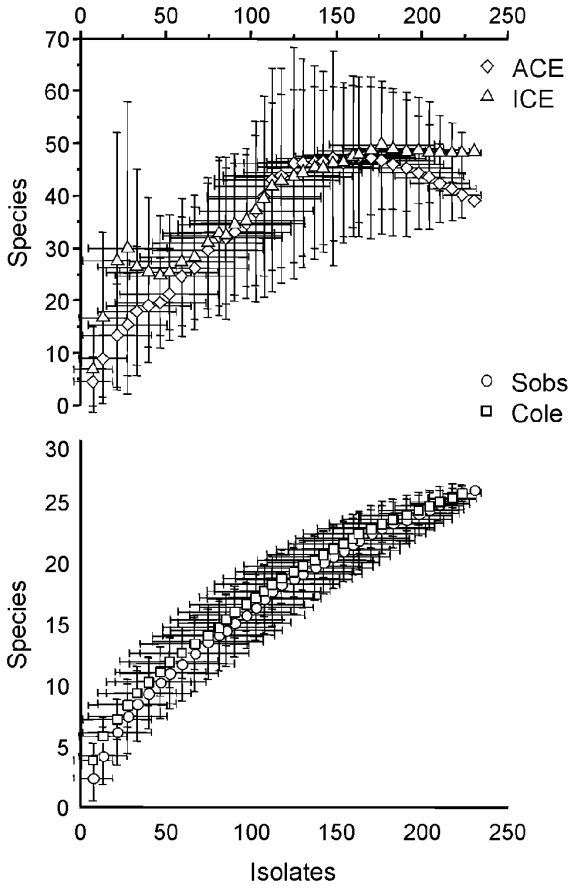

Figure 6. Yeast diversity and sample coverage estimation for the global data set. Observed species (○), Coleman estimator (□), ACE (◇) and ICE (Δ) against the number of pooled isolates. Error bars depict standard deviation.

(142 isolates). Many species of Urediniomycetes are non-pigmented but in our analysis only two species (and two isolates) were found to be non-pigmented. It is possible that the carotenoid pigments present in the basidiomycetous red yeasts, contribute to some ecological advantage in marine ecosystems.

Microbial ecology studies are deeply affected by the accuracy and rapidity of species discrimination. Yeast identification with the MSP-PCR approach proved suitable for pigmented basidiomycetous yeasts and, when used in conjunction with sequence analysis, was also effective for the characterization of non-pigmented populations. The exceptions in the identification of pigmented yeasts using MSP-PCR were *Rhodotorula nothofagi* and *Sporobolomyces roseus*, because reference fingerprints for these two species were not available in our database at the time the comparisons were made. Therefore, this approach allowed a rapid and accurate characterization of all the isolates through the formation of MSP-PCR classes that corresponded, in most cases, to distinct species. In a few cases, different profiles were obtained for members of the same species, which can be indicative of different populations within the species.

The MSP-PCR fingerprinting method shows good reproducibility because a relatively high annealing temperature is used in the PCR reaction. The main problem that can arise with PCR typing methods is the management of a large number of fingerprints in the comparative analyses. This is especially relevant for fingerprints deriving from different gels. However, since the profiles obtained by MSP-PCR are usually very clear and also because normally they are species-specific, the comparisons are relatively straightforward. This is at variance with other fingerprinting methods, like random amplified polymorphic DNA, in which the intra-specific variability is highlighted (see for example Herzberg et al. 2002). In this study, the identification module of the Gel Compar 4.1 software and the Pearson correlation coefficient were used to group the new isolates. It should be noted that a final visual confirmation of the identified profiles is necessary in order to detect eventual artefacts related to band alignment and band detection. In our laboratory an MSP-PCR database is being developed and presently 600 profiles have been entered, especially from pigmented basidiomycetous yeasts.

Studies dealing with the distribution of yeasts in aquatic environments are scarce and normally do not attempt to quantify the various populations or to analyse their occurrence in the water column. Moreover, identifications are usually based on traditional morphological/physiological methods. This study showed that yeasts, although not abundant, were frequently found. An important fraction of our isolates might be allochthonous to the marine environment because several species detected in this study have been consistently found in terrestrial habitats. The resilience of those yeasts in seawater is remarkable, since they were found not only at the upper water layers but also through the permanent thermocline and even at more deep layers withstanding high hydrostatic pressures.

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