Mar Biol (2008) 155:451–460
DOI 10.1007/s00227-008-1037-5

METHOD

Application of temperature gradient gel electrophoresis technique to monitor changes in the structure of the eukaryotic leaf-epiphytic community of *Posidonia oceanica*

F. J. Medina-Pons · J. Terrados · R. Rosselló-Móra

Received: 1 May 2008 / Accepted: 29 July 2008 / Published online: 13 August 2008

Abstract Seagrass leaves have been recognized as a suitable substratum in shallow sedimentary environments for the establishment of epiphytic communities. Microscope-based identification of species has been traditionally used to monitor changes in the composition of the eukaryotic leaf-epiphytic community of the seagrass *Posidonia oceanica* (L.) Delile. Our main goal was to adapt the temperature gradient gel electrophoresis (TGGE) barcoding technique largely used in molecular microbial ecology studies to monitor changes in the composition of *P. oceanica* epiphytic community. This molecular technique has been successful for handling large amounts of samples in a fast and reproducible manner. To that end, we applied the TGGE technique to study the epiphytic community in two different seasons and compare the results with those provided by the classical microscope approach. The results obtained with both approaches were generally consistent. The complexity of the banding pattern produced by TGGE was mirrored by the taxa richness of the community described using the classical approach. The minimum number of *P. oceanica* shoots necessary to adequately represent the composition of the eukaryotic leaf-epiphytic community was of the same order of magnitude for both techniques. Partial gene sequences of some selected bands affiliated with sequences of zoo and phytoephytic taxa. Some of them were detected using microscopy. Our results showed that TGGE is an excellent approach for comparative macrobenthic community studies that need parallel treatment of many samples at a time. To the best of our knowledge, this is the first time in which molecular barcoding techniques have been applied to the comparison of eukaryotic epiphytic communities.

Communicated by T. Reusch.

F. J. Medina-Pons (✉) · J. Terrados · R. Rosselló-Móra
Institut Mediterrani d'Estudis Avançats (CSIC-UIB)
Miquel Marqués, 21 07190 Esporles, Majorca, Spain
e-mail: vieafmp4@uib.es

Introduction

Seagrasses are a group of aquatic flowering plants distributed worldwide that can colonize depths from mean sea level down to 50 m (Duarte 1991). Seagrass leaves provide a continuously renewing substratum for the establishment of an epiphytic community (fungi, bacteria, micro- and macro-algae and sessile invertebrates) (Borowitzka et al. 2006) which contributes to the high diversity of coastal ecosystems.

Posidonia oceanica (L.) Delile is a seagrass restricted to the Mediterranean Sea where it forms extensive meadows (Procaccini et al. 2003). Its epiphytic community is a significant contributor to the biomass, primary productivity, and the nutrient cycling of the assemblage (Ballesteros 1987, Romero 1988, Lepoint et al. 2007).

While the winter leaf-epiphytic community is composed of an encrusting layer of Phaeophyta and Rhodophyta, the summer community is composed of both that encrusting layer and an erect layer of macroalgae [mostly Ceramiales (Rhodophyta)] (Van der Ben 1971, Battiato et al. 1982, Antolic 1986). In addition, the summer community is richer in taxa and the differences in taxa composition among individual shoots are lower than in the winter community (Van der Ben 1971, Ballesteros 1987).

The composition and diversity of the epiphytic community of *P. oceanica* leaves have been traditionally studied through microscopic identification by taxonomists (Ballesteros 1987, Mazzella et al. 1989, Kendrick and Lavery 2001). Molecular microbial ecology successfully uses gene

sequences of the small ribosomal subunit (16S rRNA gene sequence for prokaryotes, or 18S rRNA gene sequence in eukaryotes, hereafter abbreviated as SSU) to describe the structure and dynamics of microbial communities independent of the culture techniques (Head et al. 1998, Acinas et al. 2004). This approach, classically used for prokaryotes, has also been adapted to study the community structure of microscopic eukaryotes such as marine planktonic picoeukaryotes (Díez et al. 2001a, Díez et al. 2001b, Massana et al. 2002, Gadanho and Sampaio 2004), estuarine fungi (Heuer et al. 1997, Lefèvre et al. 2007), soil nematodes (Foucher and Wilson 2002), ruminant tract protozoa (Regensbogenova et al. 2004) and prokaryotic epiphytic community of tropical seagrasses (Uku et al. 2007).

DNA barcoding techniques like TGGE or denaturing gradient gel electrophoresis (DGGE) have been extensively applied (Ovreas et al. 1997, Nübel et al. 1999) to describe the structure of prokaryotic communities. They allow the simultaneous processing of a large number of samples and provide an estimate of diversity independently from the knowledge of the identity of the species composing the community. TGGE and DGGE are based on the separation of Polymerase chain reaction (PCR) amplified gene fragments of identical size but different in sequence along a chemical (DGGE) or thermal (TGGE) gradient (Muyzer and Smalla 1998, Muyzer 1999). Sequence separation occurs on acrylamide gels with a denaturing gradient, where each fragment migrates according to its melting point. Melting point is directly related with its guanine + cytosine (GC) content (Muyzer 1999, Gadanho and Sampaio 2004). In this regard, the complexity of the amplification pattern (i.e. the number of bands, gene fragments obtained) is directly related to the taxa composition of the community. In order to compare community structures, each band migrating with an identical velocity in TGGE has been traditionally assumed to be an operational taxonomic unit (OTU). In microbial ecology studies, each OTU is considered a different phylotype (Rosselló-Mora and López-López 2008). Due to the conservativeness of the 16S rRNA gene sequence each phylotype can be considered at least as a different species (Rosselló-Mora and Amann 2001). Given that SSU gene sequence divergence among eukaryotic species is even lower than that of prokaryotes (Eckenrode et al. 1985) each band might also represent different taxa. Eukaryotes, as prokaryotes, show paralogy in their SSU gene content. However, differences among paralogous SSU genes in eukaryotes are insignificant (Eickbush and Eickbush 2007, Ganley and Kobayashi 2007). Thus, it is highly improbable that two bands appearing separated on the same lane correspond to the same organism.

Our main objective was to evaluate the usefulness of TGGE to describe the composition changes of the eukaryotic leaf-epiphytic community of *P. oceanica*. To that end, we compare the results of TGGE with those provided by classical microscope techniques.

Materials and methods

Sample collection

The study was performed in February and September 2006 in a *P. oceanica* meadow located at a depth of 7 m in La Victoria (39° 51′ N, 3° 11′ E, Alcudia Bay, Majorca, Spain). A total of 20 *P. oceanica* shoots were haphazardly collected at each sampling date by SCUBA diving and frozen at −80°C to keep nucleic acids intact. Five (in February) and ten (in September) additional shoots were harvested and preserved in 4% formalin seawater until their processing to identify the epiphytic taxa. Five to ten shoots are considered as an adequate number to assess the taxa composition of the leaf-epiphytic community using classical microscope methods (Panayotidis and Boudouresque 1981, Ballesteros 1987).

TGGE technique

DNA isolation and purification

We expected macroalgae to be a dominant fraction of the epiphytic biota (Lepoint et al. 1999). As DNA isolation of marine algae has proven to be very difficult due to the co-isolation of polysaccharides and secondary metabolites which can inhibit PCR (Hong et al. 1997, Vidal et al. 2002), we used a modification of an aggressive DNA isolation method for soils (Zhou et al. 1996). PCR inhibiting molecules were removed from the extracted DNA by using DNeasy Plant Mini Kit (Qiagen, Ref.: 69106).

The epiphytic community of all leaves of a shoot (on average six leaves per shoot) was carefully scraped into sterile filtered seawater with a clean razor blade (we chose the whole epiphytic community of the shoot to obtain enough quantity of DNA to perform the analyses). Epiphytes were then collected by centrifugation at 2,000*g* and room temperature for 15 min. Epiphyte mass (around 1 g of wet weight) was frozen with liquid nitrogen and ground to a fine powder using 5 mm stainless steel beads (Qiagen, Ref.: 69989) and a vortex.

Disrupted cell material was suspended in 13.5 ml of extraction buffer [100 mM Tris–hydrochloric acid (Tris-HCl) (pH = 8), 20 mM ethylenediaminetetraacetic acid (EDTA) (pH = 8), 1.4 M sodium chloride (NaCl), 2% polyvinylpyrrolidone (PVP) and 2% cetyl trimethyl ammonium bromide (CTAB) (modified of Porebski et al. 1997)] and 100 µl of proteinase K (10 mg/ml). Tubes were incubated

horizontally at 37°C for 1 h with shaking. Then, 1.5 ml of 25% sodium dodecyl sulfate (SDS) was added to each tube and samples were incubated for 2 h in a 65°C water bath. Tubes were mixed by inversion each 10–15 min. Extracts were then left at room temperature for at least 20 min. After incubation, 15 ml equilibrated phenol (pH = 8) (Amersham, Ref.: US75829) was added to each sample and vigorously mixed and centrifuged for 15 min at 2,000*g* at room temperature. Aqueous supernatant was collected and mixed with 15 ml of chloroform:isoamylic alcohol (24:1), vigorously shaken, and centrifuged at 2,000*g* at room temperature for 10 min. This step was repeated at least two times, or until no interface was seen. Finally, 1/9 volume of 3 M sodium acetate (AcNa) (pH = 7) and 0.6 volume of isopropanol were added to the supernatant to precipitate DNA. To increase recovery yields, samples were stored at 4°C overnight. DNA was collected by centrifugation at 2,000*g* for 30 min at room temperature and the supernatant was discarded. Precipitated DNA was air dried at room temperature and finally dissolved in 200 µl MiliQ sterile water.

To remove PCR inhibitors, isolated DNA was further purified using DNeasy Plant Mini kit (Qiagen, Ref.: 69106) following manufacturer's instructions. Quality and quantity of purified DNA was measured in 1% agarose gel with ethidium bromide (EtBr).

PCR conditions

Eukaryotic 18S ribosomal DNA gene fragments were amplified by PCR using (rDNA)-specific primers Euk1A (5′CTG GTT GAT CCT GCC AG3′) and EukNew-516r-GC (5′CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GAC CAG ACT TGC CCT C3′) (Díez et al. 2001a). EukNew-516r-GC is a one nucleotide shorter version at the 3′ end of the previously published oligonucleotide. Comparisons of 18S rRNA gene sequence alignments in the ARB-SILVA database (http://www.arb-silva.de, Pruesse et al. 2007), using ARB software package (http://www.arb-home.de, Ludwig et al. 2004) allowed us designing a wider spectra primer set for eukaryotes. This new set allows to distinguish Rhodophyta in contrary to the previously published (Díez et al. 2001a).

A 600-pb fragment was generated with PCR using a Master Mix (Eppendorf) in a final volume of 20 µl according to manufacturer's instructions. A total of 5% dimethyl sulfoxide (DMSO) was added to increase specificity of the reaction (Sambrook and Russell 2001). The PCR program included an initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 48°C for 1 min, and extension at 72°C for 3 min, with a final extension step of 72°C for 10 min. Amplicons were visualized in a 2–3% MS-8 Agarose gel (Pronadisa, Ref.: 8066) with EtBr. "Real Escala No 2" was used as a quantification and size marker (Durviz, Ref.: RBMM2).

TGGE conditions

Polymerase chain reaction products were resolved by using the Biometra TGGE Maxi System with the procedure in accordance with the manufacturer's instructions. Acrylamide gel was prepared with 6% acrylamide/bisacrylamide (37.5:1), 8 M urea, 2% glycerol, 1× TAE (Tris–acetate–EDTA Buffer), and 20% deionized formamide. The gel was polymerized by adding 38 µl *N*,*N*,*N*,*N*-tetra-methylethylenediamine (TEMED) and 63 µl 10% ammonium persulfate (APS). The gel was let polymerizing at least for 3 h (modified of Van Dillewijn et al. 2002). About 70 ng of each PCR product were run at a constant voltage of 130 V for 17 h. For our purpose, the thermal gradient was optimal between 35 and 45°C. Finally, gels were silver-stained following a modification of a protocol of Heuer et al. 1997. The gel was fixed in 10% (v/v) ethanol plus 0.5% acetic acid (10 min). After removing fixing solution, the gel was stained with 0.2% (wt/v) silver nitrate (30 min). After four thorough washes with bi-distilled water, a freshly prepared developing solution containing sodium borohydride ($NaBH_4$) (around 0.12 g/l), 0.15% formaldehyde, and 1.5% (wt/v) sodium hydroxide (NaOH) was added to the gel. The development of the gel was stopped by adding 0.75% (wt/v) sodium carbonate solution (10 min.). Gels were finally conserved with 25% ethanol and 10% glycerol and dried. Alternatively, gels were stained by using SYBR Gold Nucleic Acid Gel Stain according to manufacturarer's instructions (Molecular Probes, Ref.: S-11494).

Band reamplification, sequencing and phylogenetic analysis

To reveal the gene sequence, some bands were excised from the freshly stained gel with SYBR gold (Molecular Probes, Ref.: S-11494) soaked in 30 µl of sterile water, and let stand at overnight at 4°C. Acrylamide pieces were disaggregated by using a plastic stab. Between 1 and 3 µl of each supernatant were used for reamplification by using the same primers and PCR conditions as above. Sequencing was performed by using the Euk 1A primer by the sequencing company Secugen SL. Partial sequences were revised and corrected with Sequencher v 4.7 (Gene Codes Corp 2006). 18S rRNA gene alignments were produced with the use of the ARB software package (http://www.arb-home.de, Ludwig et al. 2004), introducing the new almost complete sequences into a preexisting alignment available of about 208,000 single sequences (http//www.arb-silva.de, Pruesse et al. 2007). Aligned partial sequences were inserted in a

preexisting tree by the use of the ARB-Parsimony tool as implemented in the ARB package (Ludwig et al. 2004).

Optical microscope technique

Following standard methods to describe the epiphytic community of seagrasses, we studied the 10 cm apical portion of the oldest leaf (both outer and inner leaf sides) of each shoot for it has been shown to adequately represent the epiphyte community structure of the whole shoot (Vanderklift and Lavery 2000, Lepoint et al. 2007). Moreover, the species richness and biomass of epiphytes in the apical portion of seagrass leaves are usually higher that those of the basal portions (Reyes et al. 1998, Trautman and Borowitzka 1999, Lepoint et al. 2007). Random scrapings (between 3 and 5) of this apical segment were mounted on glass microscope slides for the identification of epiphytic taxa (Reyes and Sansón 1997) using an optical microscope (ZEISS AX10).

Data analysis

Temperature gradient gel electrophoresis band profiles were manually translated into a binary (presence/absence) matrix for each sampling date. All bands migrating with an identical velocity in TGGE were identified as unique OTUs (Rosselló-Mora and López-López 2008). To obtain comparable results, amplicons of different sampling dates were run together in the same gel. An averaged rarefaction curve (cumulative number of different OTUs vs. number of shoots) was constructed by using 999 permutes of the band profiles of all shoots collected in each sampling date. The minimum number of shoots required to represent the band richness of each sample was reached when the relative increase of band richness after including an additional shoot was smaller than 5%.

A non-metric multi-dimensional scaling (MDS) based on the Bray–Curtis similarity index, and an analysis of similarity (ANOSIM) was used to evaluate the differences between profiles.

Differences in number of OTUs per shoot between sampling dates were assessed by one-way ANOVA. Data were previously tested for homogeneity of variances and normality using Levene's test ($P > 0.05$) and Kolmogorov–Smirnov test ($P > 0.05$), respectively. Variances were homogeneous and data followed normal distribution.

Similar to the TGGE data analysis we calculated averaged rarefaction curves, and performed MDS, ANOSIM and one-way ANOVA analysis on the data obtained using optical microscope.

All analyses were performed using PRIMER 5 (Plymouth Routines in Multivariate Ecological Research) (Clarke and Gorley 2001), SIGMAPLOT 8.0 (SPSS Science 2002) and STATISTICA 7.1 software (StatSoft Inc 2005).

Results

A total of 43 different OTUs were identified in the complete set of samples of February and September shoots (Fig. 1). The February sample contained 30 of the total identified OTUs, whereas the September sample contained 38 of them. The number of OTUs per shoot varied between 4 and 23 in February with a mean (±SD) of 11.9 ± 5.2 OTUs per shoot. In contrast, the number of OTUs per shoot in September varied between 16 OTUs and 30 OTUs with a mean of 22.3 ± 3.9 OTUs per shoot. One-way ANOVA revealed significant differences in the number of OTUs per shoot between the February and September samples ($F_{1,36} = 48.54$, $P < 0.05$).

A total of 37 epiphytic taxa were identified by optical microscopy. The total number of epiphytic taxa identified was 10 in February shoots and 36 in September shoots. The mean number (±SD) of taxa per shoot was higher in September (14.9 ± 4.1) than in February (6 ± 2.1). One-way ANOVA revealed significant differences in taxa richness per shoot among February and September samples ($F_{1,13} = 20.32$, $P < 0.05$).

The analysis of the relative increase of OTUs richness compared to the number of shoots included in the sample increases indicated that four shoots in September and six in February were enough to represent TGGE band richness (Fig. 2a). Similarly, seven shoots in September and five shoots in February were necessary to represent the taxa richness using the optical microscope (Fig. 2b).

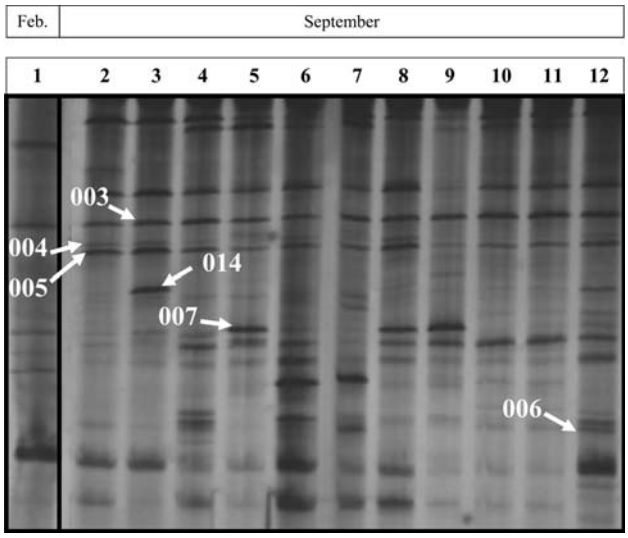

Fig. 1 Barcoding of the leaf-epiphytic community of some of the February and September shoots. *Lane 1* of the TGGE represents a February shoot and *lanes 2–12* represent September shoots. Sequenced OTUs and their codes are indicated and correspond to that listed in Table 2

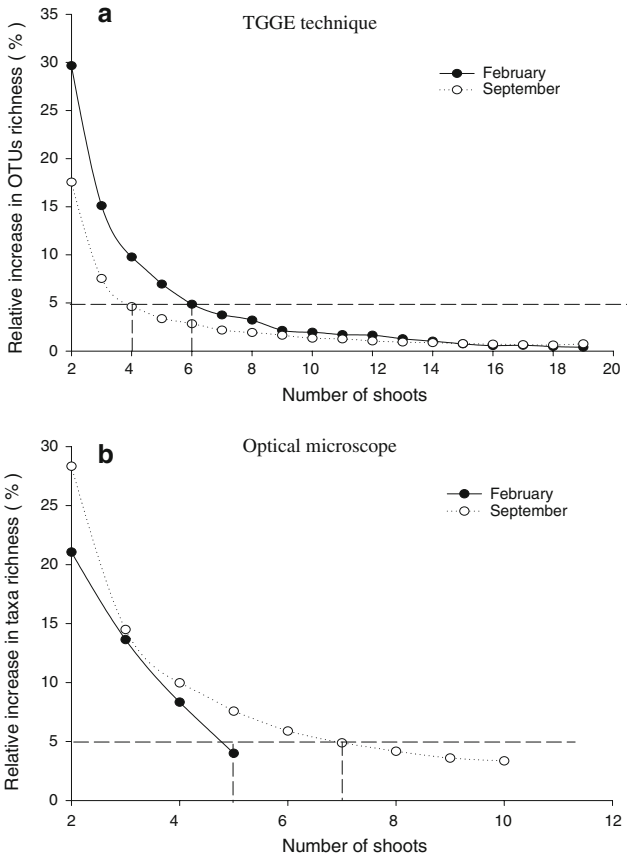

Fig. 2 Average rarefaction curves of the leaf-epiphytic community of *Posidonia oceanica* in February and September. Relative increase in (**a**) OTUs richness and (**b**) taxa richness as a function of the number of shoots. The *horizontal discontinuous line* represents the 5% level considered to indicate minimum number of shoots needed to evaluate total OTU richness or taxa richness, respectively. One sample of each sampling date was unfortunately lost while processing in the lab

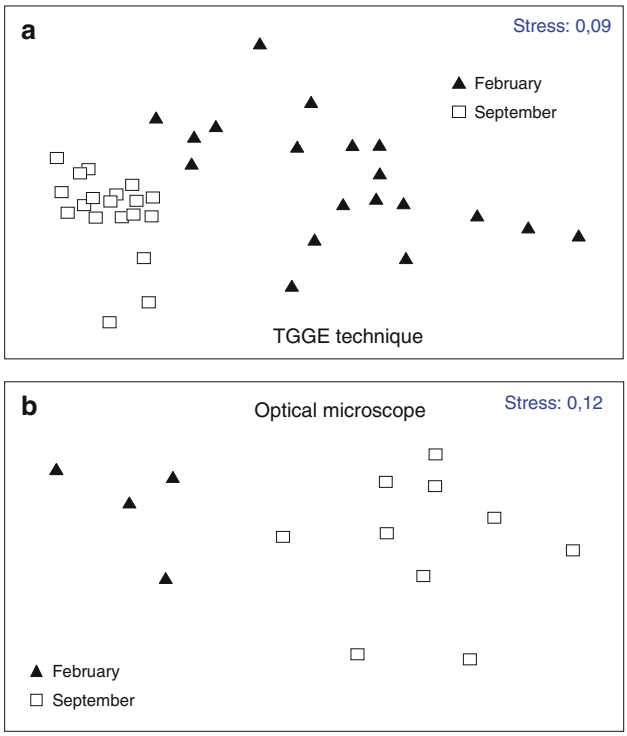

Fig. 3 MDS ordination of the leaf-epiphytic community of *Posidonia oceanica* shoots in February and September using (**a**) TGGE and (**b**) optical microscope

Multi-dimensional scaling analysis produced a clear separation between the leaf epiphytic community of February and September shoots when based both on OTUs (Fig. 3a) and on taxa richness (Fig. 3b). ANOSIM tests confirmed the significance of the differences between the two sampling dates (TGGE: $R = 0.709$, $P < 0.01$; optical microscopy: $R = 0.877$, $P < 0.01$).

A list of the epiphytic taxa identified in the February and September shoots using optical microscopy is shown in Table 1 (according to the taxonomy of Furnari et al. 2003). In general, the February epiphytic community was basically composed of Rhodophyta and Phaeophyta, whereas in September, the community was much more complex and it included Rhodophyta, Phaeophyta, Chlorophyta, and zooepiphytes. Rhodophyta and Phaeophyta showed similar taxa richness in February shoots. However, Rhodophyta (Ceramiales mainly) dominated in September shoots. Some epiphytes were found in almost all samples (brown and red encrusting algae (*Myrionema magnusii*, *Hydrolithon* sp. and *Pneophyllum* sp.) and filamentous red algae such as *Audouinella daviesii*, *Audouinella sp*., *Erythrotrichia carnea* and *Spermothamnion* sp.). However, others were mainly present either in February (brown algae *Giraudia sphacelarioides*) or in September [Ceramiales, Chlorophyta (*Phaeophila dendroides* and *Cladophora* sp) and zooepiphytes (Bryozoa and Foraminifera)]. Zooepiphytes were less present than algae in both sampling dates.

Six selected bands, representative of permanent and seasonal OTUs, were excised, reamplified and sequenced. The sequence identity (Table 2) indicated that they affiliated with algae, crustacea, bryozoa, annelida and mollusca. Some of the bands (i.e. OTU 003 and 006) represented sequences of organisms that had been detected under the microscope.

Discussion and conclusions

Our purpose was to evaluate the applicability of TGGE to the study of the epiphytic eukaryotic community of *P. oceanica* leaves comparing the results provided by this technique with those produced by taxa identification using optical microscopy. We obtained satisfactory amplification yields and clear TGGE banding profiles that could be easily compared between the two sampling dates. The TGGE

Table 1 List of leaf-epiphytic taxa found in February and September shoots (taxonomy names according to Furnari et al. 2003)

Species	Shoots													
	February					September								
	1	2	3	4	5	1	2	3	4	5	6	7	8	9	10
Bacillariophyta															
Diatoms		●	●						●			●	●	●	●
Chlorophyta															
Ulvales															
Ulvella or *Pringsheimiella* sp.								●				●			●
Cladophorales															
Chaetomorpha sp.									●						
Cladophora sp.						●		●	●	●	●	●	●	●	●
Phaeophilales															
Phaeophila dendroides							●	●		●	●	●	●	●	●
Phaeophyta															
Ectocarpales															
F. Ectocarpaceae					●										
Giraudia sphacelarioides	●	●	●	●	●							●	●	●	
Myrionema magnusii	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
Rodophyta															
Acrochaetiales															
Audouinella daviesii	●	●	●	●	●	●	●			●	●	●	●	●	●
Audouinella sp.			●		●	●						●	●	●	●
Ceramiales															
Anotrichium tenue						●									
Callithamnion corymbosum								●							
Ceramium codii						●		●			●		●		
Chondria sp.											●	●	●	●	
Chondria or *Laurencia sp.*							●					●			
Dasya sp.											●		●		
Griffithsia sp.								●				●			
Herposiphonia secunda						●		●	●		●	●		●	●
Lophosiphonia sp.											●	●	●		
Polysiphonia spp.						●		●		●	●	●	●	●	●
F. Rodomelaceae															●
Spermothamnion spp.			●		●	●	●		●	●	●	●	●	●	●
Crytptonemiales															
Hydrolithon + *Pneophyllum* spp.	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
Erythropeltidales															
Erythrotrichia carnea		●			●	●	●	●	●	●	●	●		●	●
Stylonematales															
Chroodactylon ornatum						●									
Stylonema alsidii											●				
Zooepiphytes															
Bryozoans									●	●	●	●	●	●	●
Hydrozoans					●							●			
Foraminifera							●	●	●	●	●	●	●	●	●

Table 1 continued

Species	Shoots													
	February					September								
	1	2	3	4	5	1	2	3	4	5	6	7	8	9	10
Unknown epiphytes															
Sp. 1						●			●					●	●
Sp. 2									●						
Sp. 3								●					●		
Sp. 4										●					
Sp. 5											●	●	●	●	
Sp. 6														●	
Sp. 7															●
Sp. 8														●	

Table 2 OTUs sequenced in this study. Similarity (%) is referred to the value of similarity between the OTU and the best match (according with ARB software)

Accession number	OTU	Sequence length	Best match (accession number)	Similarity (%)	Phylogenetic affiliation	Reference
EU888848	003	430	*Pneophyllum conicum* (DQ628985)	95	Rhodophyta Florideophyceae Corallinaceae	Unpublished
EU888849	004	527	*Pontoeciella abyssicola* (AY627031)	90	Crustacea Maxillopoda Copepoda	Huys et al. 2006
EU888850	005	497	*Parergodrilus heideri* (AJ310504)	81.5	Annelida Polychaeta	Rota et al. 2001
EU888851	006	550	*Bugula turrita* (AY210443)	91	Bryozoa Gymnolaemata Cheilostomata	Passamaneck and Halanych 2006
EU888852	007	541	*Brania sp.* (AY525633)	75	Annelida Polychaeta	Struck et al. 2005
EU888847	014	503	*Pomacea bridgesi* (DQ093436)	83.5	Mollusca Gastropoda	Giribet et al. 2006

technique was successful in detecting differences in the composition of the leaf epiphytic community of *P. oceanica* between the two sampling dates, and rendered comparable results to those produced by optical microscopy.

Each TGGE band represents a different SSU sequence. However, due to the highly conserved nature of the gene (Eckenrode et al. 1985), and the fact that paralogous genes may be nearly identical (Eickbush and Eickbush 2007; Ganley and Kobayashi 2007), different bands may be attributed to different taxa. In this regard, similar phylotype diversity evaluations have been previously made for microscopic eukaryotes (Rowan and Powers 1991a, b). We considered, therefore, identically migrating bands as a unique OTU, and thus each OTU may be considered a different taxon. This hypothesis has been corroborated by the excision and sequencing of six representative bands on the gel. Despite the difficulties in reamplifying and sequencing partial amplicons, the sequences retrieved were relatively clean and could undoubtedly be affiliated to known sequences in the public databases.

Temperature gradient gel electrophoresis and optical microscopy provided comparable results: (1) the number of both OTUs per shoot and epiphytic taxa per shoot were higher in September than in February; (2) the minimum number of shoots to represent OTUs richness and taxa richness were similar; (3) MDS and ANOSIM detected significant differences between the epiphytic community in the two sampling dates and (4) TGGE detected a number of common bands between the September and February shoots, and optical microscopy identified some common taxa such as *Hydrolithon* sp., *Pneophyllum sp.* and *Myrionema magnusii*. These results agree with the identification of common bands (as OTU 003 affiliating with *Pneophyllum conicum*) with sequences of organisms that appear permanent on shoots after classical optical inspection.

We found higher OTUs and taxa richness in September than in February shoots, a result that is consistent with previous microscopy studies (Antolic 1986; Ballesteros 1987). Those studies reported that taxa richness of the epiphytic community of *P. oceanica* was also higher in summer than in winter. The minimum number of shoots required to represent the richness of the epiphytic community (either 4 or 7 shoots using OTUs or identified taxa, respectively) was similar to that calculated in previous studies (from 7 to 8 shoots: Panayotidis and Boudouresque 1981; Ballesteros 1987). Furthermore, previous studies also found significant differences in the composition of the leaf epiphytic community (Antolic 1986; Ballesteros 1987) between summer and winter. Finally, the presence of a permanent group of taxa in the epiphytic community of *P. oceanica* is widely accepted (Van der Ben 1971; Romero 1988; Buia et al. 1989; Mazzella et al. 1989).

The use of optical microscopy to analyse the taxa composition of the epiphytic community produced similar results to those described previously: higher taxa richness in September shoots explained by the presence of numerous Rhodophyta (mainly filamentous Ceramiales) as Van der Ben 1971 and Antolic 1986; encrusting Rhodophyta and Phaeophyta characterized the community in February (Van der Ben 1971 and Ballesteros 1987); Chlorophyta were totally absent in winter (Tsirika et al. 2007, but see Antolic 1986).

It is clear to us that both approaches are complementary and do not give identical information. In first instance, TGGE targets all eukaryotes in the sample, including microscopic taxa, whereas the microscopy study basically focuses on macroalgae. This may explain the higher number of OTUs that TGGE renders, and could be seen as an advantage of the molecular approach. While it is true that DNA-based methods are biased (Dahllöf 2002), we have optimized a DNA aggressive purification method and PCR primer set to better reflect the real diversity of the community. On the other hand, the optical identification may be biased towards macroepiphytes and by the taxonomical expertise of the researcher.

In summary, the TGGE provides a consistent barcoding of the composition of the eukaryotic epiphytic community, and allows the simultaneous handling of large amounts of samples. Furthermore, TGGE also allows the study of a broader set of eukaryotic organisms, thus giving a better idea of the whole community and not only on specific groups. A practical benefit of the technique is that it relaxes the level of taxonomical expertise necessary to describe the diversity of the community. This goal is generally hampered by the fact that taxonomists are relatively specialized, and seldom can identify organisms that are excluded of their taxonomic expertise. Hence, fingerprinting techniques like TGGE appear as an alternative and excellent approach to the study of the structure of the epiphytic assemblage of *P. oceanica* leaves. The possibility to excise, reamplify and sequence single bands on the gel enhances the resolution power of the approach. As we have shown by selecting representative bands, the analysis of the affiliation of partial sequences by the use of the parsimony tool of the ARB program package allows the identification of putative taxa colonizing the *P. oceanica* shoots. In this regard, we could identify the sequences of some detected taxa by optical microscopy. However, as an additional benefit of the approach, we could as well detect the presence of taxa not listed in our inventory. This may help in overcoming the problems derived from the lack of expertise in morphologically recognize observable taxa.

Acknowledgments We are very grateful to the Marine Microbiology Group of IMEDEA for facilities and A. Gómez-Garreta and members of 'Departament de Productes Naturals, Biología Vegetal y Edafología' of University of Barcelona (UB) for their help with macroalgal epiphyte taxonomy. This study was funded by Ministerio de Educación y Ciencia (Acción Complementaria CTM2005-23775-E), Govern de les Illes Balears (Acció Especial de recerca, desenvolupament tecnològic i innovació, year 2006) and CSIC (Proyectos Intramurales Frontera 200530F0033 and 200530F0182). F.J.M.P would like to thank 'Conselleria de Economia, Hisenda e Innovació' of Balearic Government for a PhD grant (FPI05) that supported this work. All experiments done in this study comply with the current laws of Spain.

References

Acinas SG, Klepac-Ceraj V, Hunt DE, Pharino C, Ceraj I, Distel DL et al (2004) Fine-scale phylogenetic architecture of a complex bacterial community. Nature 430(6999):551–554. doi:10.1038/nature02649

Antolic B (1986) Epiphytic flora on leaves of *Posidonia oceanica* (L.) Delile from the area of Dubrovnik (South Adriatic). Acta Adriat 27(1-2):37–49

Ballesteros E (1987) Estructura i dinàmica del poblament algal de les fulles de *Posidonia oceanica* (L.) Delile als herbeis de Tossa de Mar (Girona). Bullt Inst Cat Hist Nat 54:13–30

Battiato A, Cinelli F, Cormaci M, Furnari G, Mazzella L (1982) Studio preliminare della macroflora epifita della Posidonia oceanica (L.) Delile di una prateria di Ischia (Golfo di Napoli) (Potamogetonaceae, Helobiae). Nat Sicil 1:15–27

Borowitzka MA, Lavery PS, Van Keulen M (2006) Epiphytes of seagrasses. In: Larkum AWD, Orth RJ, Duarte CM (eds) Seagrasses: biology, ecology and conservation. Springer, Dordrecht, pp 441–461

Buia MC, Cormaci M, Furnari G, Mazzella L (1989) Posidonia oceanica off Capo Passero (Sicily, Italy): leaf phenology and leaf algal epiphytic community. GIS. Posidonie Publ Fr, 2: 127–143

Clarke KR, Gorley RN (2001) Primer (Plymouth Routines In Multivariate Ecological Research) V5: User Manual/Tutorial. Primer-E Ltd, Plymouth

Dahllöf I (2002) Molecular community analysis of microbial diversity. Curr Opin Biotechnol 13:213–217. doi:10.1016/S0958-1669(02)00314-2

Díez B, Pedrós-Alió C, Marsh TL, Massana R (2001a) Application of denaturing gradient gel electrophoresis (DGGE) to study the diversity of marine picoeukaryotic assemblages and comparison of DGGE with other molecular techniques. Appl Environ Microbiol 67(7):2942–2951. doi:10.1128/AEM.67.7.2942-2951.2001

Díez B, Pedrós-Alió C, Massana R (2001b) Study of genetic diversity of eukaryotic picoplankton in different oceanic regions by small-subunit rRNA gene cloning and sequencing. Appl Environ Microbiol 67(7):2932–2941. doi:10.1128/AEM.67.7.2932-2941.2001

Duarte CM (1991) Seagrass depth limits. Aquat Bot 40(4):363–377. doi:10.1016/0304-3770(91)90081-F

Eckenrode VK, Arnold J, Meagher RB (1985) Comparison of the nucleotide sequence of soybean 18S rRNA with the sequences of other small-subunit rRNAs. J Mol Evol 21(3):259–269. doi:10.1007/BF02102358

Eickbush TH, Eickbush DG (2007) Finely orchestrated movements: evolution of the ribosomal RNA genes. Genetics 175:477–485. doi:10.1534/genetics.107.071399

Foucher A, Wilson M (2002) Development of a polymerase chain reaction-based denaturing gradient gel electrophoresis technique to study nematode species biodiversity using the 18s rDNA gene. Mol Ecol Notes 2(1):45–48. doi:10.1046/j.1471-8286.2002.00141.x

Furnari G, Giaccone G, Cormaci M, Alongi G, Serio D (2003) Biodiversità marina delle coste italiane. Catalogo del Macrofitobenthos. Biol Mar Medit 10(1):1–483

Gadanho M, Sampaio JP (2004) Application of temperature gradient gel electrophoresis to the study of yeast diversity in the estuary of the Tagus river, Portugal. FEMS Yeast Res 5(3):253–261. doi:10.1016/j.femsyr.2004.09.001

Ganley ARD, Kobayashi T (2007) Highly efficient concerted evolution in the ribosomal DNA repeats: total rDNA repeat variation revealed by whole-genome shotgun sequence data. Genome Res 17:184–191. doi:10.1101/gr.5457707

Giribet G, Okusu A, Lindgren AR, Huff SW, Schrödl M, Nishiguchi MK (2006) Evidence for a clade composed of molluscs with serially repeated structures: monoplacophorans are related to chitons. Proc Natl Acad Sci USA 103(20):7723–7728

Head IM, Saunders JR, Pickup RW (1998) Microbial evolution, diversity, and ecology: a decade of ribosomal RNA analysis of uncultivated microorganisms. Microb Ecol 35(1):1–21. doi:10.1007/s002489900056

Heuer H, Krsek M, Baker P, Smalla K, Wellington EMH (1997) Analysis of actinomycete communities by specific amplification of genes encoding 16S rRNA and gel-electrophoretic separation in denaturing gradients. Appl Environ Microbiol 63(8):3233–3241

Hong YK, Sohn CH, Lee KW, Kim HG (1997) Nucleic acid extraction from seaweed tissues for polymerase chain reaction. J Mar Biotechnol 5(2–3):95–99

Huys R, Llewelyn-Hughes J, Olson PD, Nagasawa K (2006) Small subunit rDNA and Bayesian inference reveal *Pectenophilus ornatus* (Copepoda *incertae sedis*) as highly transformed Mytilicolidae, and support assignment of Chondracanthidae and Xarifiidae to Lichomolgoidea (Cyclopoida). Biol J Linn Soc Lond 87:403–425. doi:10.1111/j.1095-8312.2005.00579.x

Kendrick GA, Lavery PS (2001) Assessing biomass, assemblage structure and productivity of algal epiphytes on seagrasses. In: Short FT, Coles RG, Short CA (eds) Global Seagrass Research Methods. Elsevier, Amsterdam, The Netherlands, pp 199–222

Lefèvre E, Bardot C, Noel C, Carrias JF, Viscogliosi E, Amblard C et al (2007) Unveiling fungal zooflagellates as members of freshwater picoeukaryotes: evidence from a molecular diversity study in a deep meromictic lake. Environ Microbiol 9(1):61–71. doi:10.1111/j.1462-2920.2006.01111.x

Lepoint G, Havelange S, Gobert S, Bouquegneau JM (1999) Fauna vs flora contribution to the leaf epiphytes biomass in a *Posidonia oceanica* seagrass bed (Revellata Bay, Corsica). Hydrobiologia 394:63–67. doi:10.1023/A:1003557303904

Lepoint G, Jacquemart J, Bouquegneau JM, Demoulin V, Gobert S (2007) Field measurements of inorganic nitrogen uptake by epiflora components of the seagrass *Posidonia oceanica* (Monocotyledons, Posidoniaceae). J Phycol 43:208–218. doi:10.1111/j.1529-8817.2007.00322.x

Ludwig W, Strunk O, Westram R, Richter L, Meier H Yadhukumar, Buchner A, Lai T, Steppi S, Jobb G, Forster W, Brettske I, Gerber S, Ginhart AW, Gross O, Grumann S, Hermann S, Jost R, Konig A, Liss T, Lussmann R, May M, Nonhoff B, Reichel B, Strehlow R, Stamatakis A, Stuckmann N, Vilbig A, Lenke M, Ludwig T, Bode A, Schleifer KH (2004) ARB: a software environment for sequence data. Nucl Acids Res 32: 1363–1371. http://www.arb-home.de/

Massana R, Guillou L, Diez B, Pedros-Alio C (2002) Unveiling the organisms behind novel eukaryotic ribosomal DNA sequences from the ocean. Appl Environ Microbiol 68(9):4554–4558. doi:10.1128/AEM.68.9.4554-4558.2002

Mazzella L, Scipione MB, Buia MC (1989) Spatio-temporal distribution of algal and animal communities in a *Posidonia oceanica* meadow. Mar Ecol Evol Perspect 10:107–129

Muyzer G (1999) DGGE/TGGE a method for identifying genes from natural ecosystems. Curr Opin Microbiol 2(3):317–322. doi:10.1016/S1369-5274(99)80055-1

Muyzer G, Smalla K (1998) Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. Antonie Van Leeuwenhoek 73(1):127–141. doi:10.1023/A:1000669317571

Nübel U, Garcia-Pichel F, Kuhl M, Muyzer G (1999) Quantifying microbial diversity: Morphotypes, 16S rRNA genes, and carotenoids of oxygenic phototrophs in microbial mats. Appl Environ Microbiol 65(2):422–430

Ovreas L, Forney L, Daae FL, Torsvik V (1997) Distribution of bacterioplankton in meromictic Lake Saelenvannet, as determined by denaturing gradient gel electrophoresis of PCR-amplified gene fragments coding for 16S rRNA. Appl Environ Microbiol 63(9):3367–3373

Panayotidis P, Boudouresque CF (1981) Vegetation marine de l'Ile de Port-Cros (Parc National) XXI. Aire Minimale et patchiness de la flore epiphyte des feuilles de Posidonia oceanica. Trav Sci Parc Nation Port-Cros 7:71–84

Passamaneck Y, Halanych KM (2006) Lophotrochozoan phylogeny assessed with LSU and SSU data: evidence of lophophorate polyphyly. Mol Phylogenet Evol 40:20–28. doi:10.1016/j.ympev.2006.02.001

Porebski S, Bailey LG, Baum BR (1997) Modification of a CTAB DNA extraction protocol for plants containing high polysaccharide and polyphenol components. Plant Mol Biol Rep 15(1):8–15. doi:10.1007/BF02772108

Procaccini G, Buia MC, Gambi MC, Pérez M, Pergent G, Pergent-Martini C et al (2003) The seagrasses of the Western Mediterranean. In: Green EP, Short FT (eds) World Atlas of Seagrasses. UNEP World Conservation Monitoring Centre. University of California Press, Berkeley, pp 48–58

Pruesse E, Quast C, Knittel K, Fuchs B, Ludwig W, Peplies J et al (2007) SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. Nucleic Acids Res 35(21):7188–7196. doi:10.1093/nar/gkm864

Regensbogenova M, Pristas P, Javorsky P, Moon-van der Staay SY, van der Staay GWM, Hackstein JHP et al (2004) Assessment of ciliates in the sheep rumen by DGGE. Lett Appl Microbiol 39(2):144–147. doi:10.1111/j.1472-765X.2004.01542.x

Reyes J, Sansón M (1997) Temporal distribution and reproductive phenology of the epiphytes on *Cymodocea nodosa* leaves in the Canary Islands. Bot Mar 40(3):193–201

Reyes J, Sansón M, Afonso-Carrillo J (1998) Distribution of the epiphytes along the leaves of *Cymodocea nodosa* in the Canary Islands. Bot Mar 41(6):543–551

Romero J (1988) Epífitos de las hojas de *Posidonia oceanica*: variaciones estacionales y batimétricas de biomasa en la pradera de las islas Medes (Girona). Oecol Aquat 9:19–25

Rosselló-Mora R, Amann R (2001) The species concept for prokaryotes. FEMS Microbiol Rev 25(1):39–67

Rosselló-Mora R, López-López A (2008) The least common denominator—species or OTU's. In: Zengler K (ed) Accessing uncultivated microorganisms. From the environment to organisms and genomes and back. ASM press, Washington

Rota E, Martin P, Erséus C (2001) Soil-dwelling polychaetes: enigmatic as ever? Some hints on their phylogenetic relationships as suggested by a maximum parsimony analysis of 18S rRNA gene sequences. Contrib Zool 70(3):127–138

Rowan R, Powers DA (1991a) A molecular genetic identification of zooxanthellae and the evolution of animal-algal symbioses. Science 251:1348–1351. doi:10.1126/science.251.4999.1348

Rowan R, Powers DA (1991b) Molecular genetic identification of symbiotic dinoflagellates (zooxanthellae). Mar Ecol Prog Ser 71:65–73. doi:10.3354/meps071065

Sambrook J, Russell DW (2001) In vitro amplification of DNA by PCR. Molecular cloning: a laboratory manual, 3rd edn. vol II, Chapter 3, Section 8.1 Cold Spring Harbor Laboratory Press, New York

Sequencher v 4.7 (2006) Gene Codes Corporation, Ann Arbor, Michigan. http://www.sequencher.com

Science SPSS (2002) SigmaPlot 8.0 User's Guide, USA. http://www.sigmaplot.com

StatSoft Inc (2005) STATISTICA (data analysis software system), version 7.1. http://www.statsoft.com

Struck TH, Purschke G, Halanych KM (2005) A scaleless scale worm: Molecular evidence for the phylogenetic placement of *Pisione remota* (Pisionidae, Annelida). Mar Biol Res 1(4):243–253. doi:10.1080/17451000500261951

Trautman DA, Borowitzka MA (1999) Distribution of the epiphytic organisms on *Posidonia australis* and *P.sinuosa*, two seagrasses with differing leaf morphology. Mar Ecol Prog Ser 179:215–229. doi:10.3354/meps179215

Tsirika A, Skoufas G, Haritonidis S (2007) Seasonal and bathymetric variations in epiphytic macroflora on *Posidonia oceanica* (L.) Delile leaves in the National Marine Park of Zakynthos (Greece). Mar Ecol (Berl) 28(Suppl 1):146–153

Uku J, Bjork M, Bergman B, Diez B (2007) Characterization and comparison of prokaryotic epiphytes associated with three east african seagrasses. J Phycol 43(4):768–779. doi:10.1111/j.1529-8817.2007.00371.x

Van der Ben D (1971) Les épiphytes des feuilles de *Posidonia oceanica* Delile sur les côtes françaises de la Méditerranée. Mem Inst R Sci Nat Belg 168:1–101

Van Dillewijn P, Villadas PJ, Toro N (2002) Effect of a *Sinorhizobium meliloti* strain with a modified putA gene on the rhizosphere microbial community of alfalfa. Appl Environ Microbiol 68(9):4201–4208. doi:10.1128/AEM.68.9.4201-4208.2002

Vanderklift MA, Lavery PS (2000) Patchiness in assemblages of epiphytic macroalgae on *Posidonia coriacea* at a hierarchy of spatial scales. Mar Ecol Prog Ser 192:127–135. doi:10.3354/meps192127

Vidal R, Meneses I, Smith M (2002) Enhanced DNA extraction and PCR amplification of SSU ribosomal genes from crustose coralline algae. J Appl Phycol 14(3):223–227. doi:10.1023/A:1019975409640

Zhou JZ, Bruns MA, Tiedje JM (1996) DNA recovery from soils of diverse composition. Appl Environ Microbiol 62(2):316–322