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ORIGINAL PAPER

Bacterial composition of microbial mats in hot springs in Northern Patagonia: variations with seasons and temperature

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Abstract Seasonal shifts in bacterial diversity of microbial mats were analyzed in three hot springs (39–68 °C) of Patagonia, using culture-independent methods. Three major bacterial groups were detected in all springs: Phyla Cyanobacteria and Bacteroidetes, and Order Thermales. Proteobacteria, Acidobacteria and Green Non-Sulfur Bacteria were also detected in small amounts and only in some samples. Thermophilic filamentous heterocyst-containing *Mastigocladus* were dominant Cyanobacteria in Porcelana Hot Spring and Geyser, and *Calothrix* in Cahuelmó, followed by the filamentous non-heterocyst *Leptolyngbya* and *Oscillatoria*. Bacteroidetes were detected in a wide temperature range and their relative abundance increased with decreasing temperature in almost all samples. Two *Meiothermus* populations with different temperature optima were found. Overall, fingerprinting analysis with universal bacterial primers showed high similarities within each hot spring despite differences in temperature. On the other hand, Cahuelmó Hot Spring showed a lower resemblance among samples. Porcelana Hot Spring and Porcelana Geyser were rather similar to each other, possibly due to a common geological substrate given their geographic proximity. This was even more evident with specific cyanobacterial primers. The different geological substrate and the seawater influence in Cahuelmó might have caused the differences in the microbial community structure with the other two hot springs.

Keywords Hot spring · Northern Patagonia · DGGE fingerprint · Microbial mat · Bacterial diversity

Introduction

Hot springs with source water temperatures above 40 °C can be found all along the Southern Andes (Lahsen 1988). Most of the previously studied hot springs and geysers are located in the Atacama Desert and Altiplano (18°–27° S, Northern Chile). Most of these studies focused on geological and geochemical features (Jones and Renaut 1997; Glennon and Pfaff 2003; Fernandez-Turiel et al. 2005; Tassi et al. 2010), on the resistance to ultraviolet radiation (Phoenix et al. 2006) or on development of an infrared sensing system to map temperature in the mats (Dunckel et al. 2009).

The Patagonian Andes (39°–47° South latitude) are also volcanically active and, as a consequence, many hot springs are scattered throughout the fjords and forests. Many of these have remained unknown until recently and most have not been studied at all (Hauser 1989). The region is covered by Valdivian temperate rainforest, a unique ecosystem considered to be a hotspot for biodiversity of plants and vertebrates (Arroyo et al. 2004). Likewise, hot springs harbor a diversity of thermophilic microbial lineages, with potential interest for evolution, biogeography, microbial ecology studies (Whitaker et al.

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2003; Papke et al. 2003; Miller et al. 2007), as well as for biotechnology (Brock 1997; Klatt et al. 2011). Moreover, a better knowledge of these systems will allow better sustainable management of the ecosystems (Smith-Ramírez 2004).

One aspect that has received little attention is the seasonal variations in the hot spring communities, maybe assuming that the supposedly stable physico-chemical condition of the source water would attenuate seasonal changes. The few studies carried out, however, did show significant seasonal changes (Ferris and Ward 1997; Lacap et al. 2007). Here, we compared the composition of the mat communities at two different seasons.

Finally, hot springs can be considered as biogeographic islands (Papke et al. 2003) and useful to explore the potential existence of biogeography in microorganisms (Martiny et al. 2006). Here, we analyzed three different hot springs at different distances from each other.

Different molecular methods have been used to analyze the composition of microbial mats in hot springs, for example, in Yellowstone (USA, Ward et al. 1998), Kamchatka (Russia, Perevalova et al. 2008), Seltun and Hveradalir (Iceland, Aguilera et al. 2010), Boekleung (Thailand, Portillo et al. 2009), and El Coquito (Colombia, Bohorquez et al. 2012). DGGE has been effective in allowing efficient comparison of different communities (Nocker et al. 2007) including some studies of hot springs (Perevalova et al. 2008; Portillo et al. 2009). Here we used DGGE with two different sets of primers: general for bacteria and specific for cyanobacteria and chloroplasts.

Cahuelmó and Porcelana Hot Springs have only received a few sporadic visits determining a few physico-chemical parameters (Hauser 1989; Waring 1965), but no microbiological studies have been carried out. The Porcelana Geyser, in turn, has not been described at all. The purpose of the present study was to obtain information on the bacterial community composition and its changes in space and time.

Materials and methods

Sampling sites

Microbial mat samples were obtained from hot springs Porcelana (42° 27′ 29.1″S–72° 27′ 39.3″W), and Cahuelmó (42° 15′ 11.8″S–72° 22′ 4.4″W) in June (austral winter) and December (austral summer) 2009. Porcelana Geyser (42° 24′ 51″S/72° 29′ 02.2″W) was sampled only in June 2009 due to the trail being impassable in December. These hot springs are located in the surroundings of Comau and Cahuelmó Fjords, North Chilean Patagonia. The linear distance between hot springs is 5.1 km between Porcelana Hot Spring and Porcelana Geyser, 25.4 km between Porcelana and Cahuelmó, and 20.6 km between Cahuelmó and Porcelana Geyser (Fig. 1).

Cahuelmó Hot Spring is in the coast of Cahuelmó Fjord (at sea level), exposed to brackish water influence (salinity 3 %) and wind. This system is located in a metamorphic rock complex, closer to the Andes mountain chain, so it is rich in metallic minerals and elements such as pyrite, polonium, magnetite, and chalcopyrite (Duhart et al. 2000).

Porcelana Hot Spring and Porcelana Geyser are located in Huequi Peninsula. They are in an area of shallow depth geothermic events whose outflow pours over volcanic rocks originated in the Quaternary Period and are encircled by active Quaternary volcanoes such as the Huequi Volcano (Duhart et al. 2000). The volcanic rocks in this region are formed mainly by silicates and carbonates and are rich in metallic minerals and elements such as pyrite, chalcopyrite, arsenopyrite, and Antimony (Fortey et al. 1992).

Four points were sampled per transect. The different points along the transects were defined by the differences in water temperature encountered along the gradient. Thus, some sampling points were slightly different in the June and December visits.

Environmental characteristics of hot springs and sample collection

Temperature and conductivity were determined using a conductivity meter (Oakton, model 35607-85); dissolved oxygen was measured using a dissolved oxygen probe (YSI, model 550I), and pH using pH-indicator paper (Merck, Germany). Salinity of Cahuelmó Fjord was measured using a refractometer (ATAGO S/Mill-E, Japan). Two-milliliter samples of microbial mats at each selected temperature point (in triplicate biological replicates) were collected in sterile plastic cryovials, and frozen in liquid nitrogen in situ for DNA analyses.

Nucleic acid extraction, PCR and DGGE

Samples were thawed and approximately 200 μl of microbial mat was added to a bead beating tube with different glass beads for rapid and thorough homogenization. Nucleic acids were extracted using a modified phenol: chloroform: IAA protocol, and purified using Amicon Ultra-15 (Millipore, MA, USA). Resulting total DNA was measured in a Nanodrop (Thermo Scientific, DE, USA). The 16S rRNA gene was amplified using universal bacterial primers (358Fgc—907R) (Muyzer et al. 1995), as described by Schauer et al. (2003); cyanobacterial-specific primers [CYA106Fgc—CYA781R(a) and CYA781R(b)], as described by Díez et al. (2007). Each PCR reaction contained 1.25 μl of 10 mM primer (each), 0.5 μl of

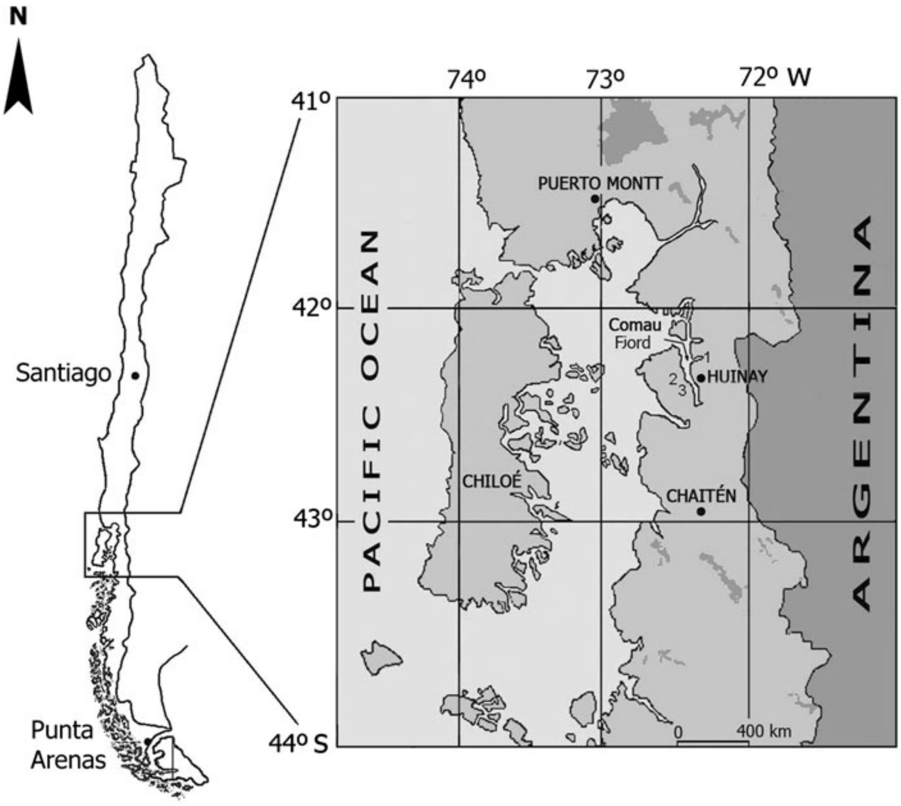

Fig. 1 Map of Los Lagos District, Chile. Hot springs included in the present study are indicated by numbers *1* Cahuelmó Hot Spring, *2* Porcelana Geyser, *3* Porcelana Hot Spring

deoxynucleoside triphosphates (dNTP Mix 10 mM, Applied Biosystems), 2.5 µl of PCR Flexi colourless buffer 1X, 0.75 µl of $MgCl_2$ solution 25 mM, 1.25 µl of BSA 3 mg/ml, 0.125 U polymerase (HotStar Taq polymerase, Qiagen), and a 2-microliter aliquot of the DNA template for each PCR reaction. All reactions were made up to 25 µL with DNAse/RNAse free H_2O (ultraPURE, Gibco).

DGGE was carried out using a DGGE-2000 system (CBS Scientific Company) as described by Muyzer et al. (1995). All DGGEs were carried out casting a 6 % polyacrylamide gel, with gradients of DNA-denaturant agent of 50–75 % for universal bacterial primers, and 45–75 % for cyanobacterial specific primers. Approximately 800 ng of PCR product was loaded for each sample and the gel was run at 100 V for 16 h at 60 °C in 1× TAE buffer [40 mM Tris (pH 7.4), 20 mM sodium acetate, and 1 mM EDTA]. Gels were stained with SybrGold (Molecular Probes) for 30 min, rinsed with MilliQ water, removed from the glass plate to a UV transparent gel scoop, and visualized with UV in a Fluor-S MultiImager (Bio-Rad) with the Multi-Analyst software (Bio-Rad), and recorded. Image analysis was performed using the Quantity One 1D Analysis software (Bio-Rad, CA, USA). Bray–Curtis similarity index was calculated based on DGGE fingerprints of hot springs, using the Primer 6 Software (V 6.1.2).

The 43 most intense bands from both bacterial and cyanobacterial DGGE gels (25 and 18 bands, respectively) were excised, reamplified, and sequenced. Closest relatives were determined with BLAST. The 16S rDNA gene sequences from this study have been deposited in the European Nucleotide Archive under accession numbers HE979738 to HE979757. Sequence analysis was carried out using the Chromas Software V 2.33 and the BioEdit Sequence Alignment Editor software V 7.0.5.3. Phylogenetic trees were constructed using the MEGA Software V 5.01. Diversity graphics were constructed using the OriginPro 8 SR0V. 8.0724 (MA, USA), summing up the intensity of bands belonging to the same bacterial phylum and calculating the proportion of each phylum in the total bacterial community.

Results and discussion

Physico-chemical characteristics

The three hydrothermal systems studied showed a continuous water discharge with temperature ranging from 45 to 68 °C. In all cases temperature decreased and oxygen increased downstream, as expected (Table 1). Both temperature and oxygen values at the sources were very similar

Table 1 Physicochemical characteristics of the three hot springs

Hot spring	Sample	T (°C)	Dissolved oxygen (%)	Conductivity (µs/cm)	pH	Publication
Cahuelmó	C1w	57.8	13.3	2150	7	This study
	C2w	54.8	15.4	2120	6	
	C3w	48	35	2080	5	
	C4w	41.7	38.4	2090	5	
	C1s	58.7	11.2	2621	6	This study
	C5s	46.6	17.8	2652	6	
	C6s	45.1	33.5	–	6	
	C2s	–	64.4	2631	6	
	*	84	–	256	8.25	Hauser (1989)
	*	58	–	–	–	Waring (1965)
Porcelana	P1w	52.4	39.4	1086	6	This study
	P2w	48.6	51	972	6	
	P3w	43.7	55.4	952	6	
	P4w	39.5	61.2	905	6	
	P7s	45.6	41.9	1092	5	This study
	P6s	41.5	46.1	1071	6	
	P5s	40.7	47.7	1082	5	
	P2s	39.7	42.9	1113	6	
	*	60	–	–	7	Hauser (1989)
Porcelana Geyser	F1w	68.3	40.2	1224	5	This study
	F2w	55	26.5	1491	6	
	F3w	50.8	38.3	2030	6	
	F4w	40.8	44.3	2300	6	

Measurements of Porcelana and Cahuelmó were made in winter (*w*) and summer (*s*) in year 2009. Measurements of Porcelana Geyser were made only in June 2009. The available data from the literature are also shown

* The authors reported no information on the exact point measured. It was assumed they measured the source of the springs

in winter and summer in Cahuelmó and Porcelana Geyser. In Porcelana hot spring there was a difference of seven degrees between the two seasons. This difference in temperature was due to the presence of several sources at this place that are not always active and, thus, the source sampled in June could not be sampled in December. Porcelana Geyser showed the highest temperature (68.3 °C) followed by Cahuelmó (~58 °C) and Porcelana (45.6–52.4 °C). In fact, temperature at the source in Porcelana Geyser was 99 °C, but it cooled down quickly before reaching the first microbial mats that was possible to sample. Temperature in Porcelana Hot Spring was higher during winter than in summer, with almost 7 °C difference in the hottest point sampled. Dissolved oxygen was lower in Cahuelmó (11.2–13.3 % saturation) than at the two other springs (around 40 % saturation).

Hauser (1989) provided data such as temperature, conductivity, or pH for 20 different hot springs in the same area, with temperatures ranging from 18 to 84 °C, while Waring (1965) reported some data from 9 hot springs in the area, covering a temperature range between 22 and 60 °C. Table 1 summarizes the information from those studies, together with the data obtained in this study. In the case of Cahuelmó, Waring (1965) reported a temperature essentially identical to that found in our study. However, in a study published two decades later, Hauser (1989) reported 84 °C in Cahuelmó. The temperature difference in these two decades is approximately 26 °C. Hauser (1989) also reported that the Porcelana Hot Spring was at 60 °C, 8 °C higher than that reported in our study. As mentioned above, the Porcelana Geyser had not been studied earlier.

Conductivity and pH only showed minor differences between seasons and among sampling points. The pH was slightly acidic (around 5–6) or neutral. The pH reported by Hauser (1989) was also higher than the pH detected in this study in Cahuelmó and Porcelana (Table 1) and was measured by electrometric determination. On the contrary, conductivity was ten times higher in Cahuelmó in the present study than in Hauser (1989). Hauser reported no conductivity data for Porcelana. Conductivity was higher in Cahuelmó (above 2000 µS cm^{-1}) than in the other two springs (around 1000 µS cm^{-1}). Sulfate ranged between 0.43 and 7.89 mg L^{-1} in Cahuelmó, between 40.6 and 66.9 mg L^{-1} in Porcelana, and between 46.1 and 61.7 mg L^{-1} in Porcelana Geyser. Clearly, the two Porcelana springs were about 10 times richer in sulfate than Cahuelmó.

These results indicate that these geothermal systems experience substantial environmental shifts, probably related to the variable volcanic activities in the area.

Bacterial assemblages

Comparison among bacterial communities at the three hot springs

A dendrogram comparing DGGE fingerprints from all samples showed differences among all three hot springs (<50 % Bray–Curtis similarity). All samples from Porcelana Geyser formed a separate cluster, higher than 55 % Bray–Curtis similarity. Porcelana Hot Spring winter samples also formed a separate cluster (Bray–Curtis index larger than 55 %). However, the Porcelana Hot Spring samples collected in summer were more different from each other, in particular points P2s and P5s, within this spring and season (Fig. 2a). Cahuelmó samples showed the largest differences with temperature and season, resulting in a complex pattern of clusters when analyzing its DGGE fingerprints. This is probably due to human influence, since Cahuelmó is the only hot spring of the three used by tourists. Even though the human presence is very low, and only in summer, tourists change the water courses to take hot baths in pools nearby, disrupting the stability and development of microbial communities in the mats.

Bacterial community composition at the three hot springs

All three hot springs showed a dominance of Phyla Cyanobacteria (23.5 % on average), Bacteroidetes (21.5 %), and the Order Thermales (19.6 %) (Fig. 2b). Combined they accounted for 71 % of total band intensity in Porcelana Geyser, 58 % in Porcelana, and 65 % in Cahuelmó. The remaining groups detected, Acidobacteria, Green Non-sulfur Bacteria, and Beta-, Gamma-, and Epsilon Proteobacteria, were present in small amounts and not in all samples. In general, the retrieved sequences had a higher phylogenetic resemblance with uncultured members of these phyla rather than with cultured species. As expected, most of the closest relatives in GenBank had originated in high-temperature environments. In Porcelana Geyser, only 3.7 % of the total band intensity could not be assigned to any group. The percentage of undetermined band intensity in Porcelana was 32.5 % in winter and 22.3 % in summer. In Cahuelmó percentages were 16.1 % in winter and 8.7 % in summer (Fig. 2b).

Phylum Cyanobacteria

Cyanobacteria are usually the dominant phototrophic organisms in non-acidic hot environments (Ferris et al. 1996; Ward et al. 1998; Roeselers et al. 2007; Klatt et al. 2011; Miller et al. 2007). In our study, the presence or

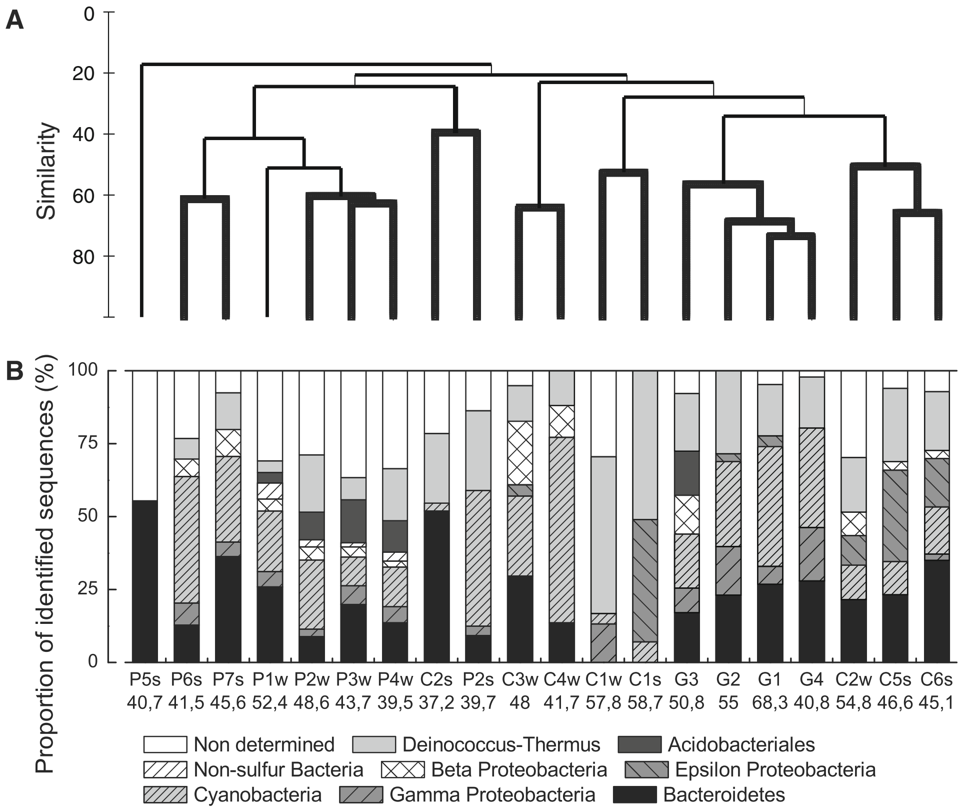

Fig. 2 **a** Dendrogram comparing all samples according to the relative band intensities of the different bacterial groups. The *vertical axis* is shown as the Bray–Curtis similarity distances. **b** Relative composition of each bacterial assemblage based on 16S ribosomal RNA DGGE band intensities. The letters *w* and *s* in sample code indicate winter and summer, respectively. Temperature (°C) appears below sampled points

dominance of Cyanobacteria was confirmed in all samples except for P5s at Porcelana Hot Spring. This sample had the largest percent of unaccounted band intensity (46 %) and only Bacteroidetes sequences could be retrieved (see below). Perhaps the absence of Cyanobacteria sequences was related to this large percent of unidentified DGGE bands (Fig. 2b). On average, Cyanobacteria accounted for 29 % of total band intensity in Porcelana Geyser. In Cahuelmó their contribution decreased from 26.6 % in winter to 9.3 % in summer. On the contrary, in Porcelana, it increased from 16.9 % in winter to 29.8 % in summer, similar to Porcelana Geyser.

Our analysis of 16S rRNA genes with general bacterial primers detected several bands affiliated to Cyanobacteria which were separated in two major groups: filamentous heterocystous (HCYA) and filamentous non-heterocystous cyanobacteria (NHCYA) (Fig. 3a). NHCYA were present in all samples, while HCYA were absent from six samples, five from Cahuelmó and one from Porcelana Hot Spring.

Cyanobacterial specific primers were used to analyze these important organisms in more detail. Unicellular cyanobacteria not retrieved with the general bacterial primers were found using the specific primers. These unicellular *Synechococcus*-type sequences only appeared in samples C1w and C5s from Cahuelmó Hot Spring (Fig. 3c).

In general, when using the specific primers, we were rather successful in identifying most of the important DGGE bands present at the Porcelana Geyser and Hot Spring fingerprints, with an average of only 0.4 and 4 % of the total bands not identified, respectively. In Cahuelmó, however, a substantial percent (29 % approx.) could not be

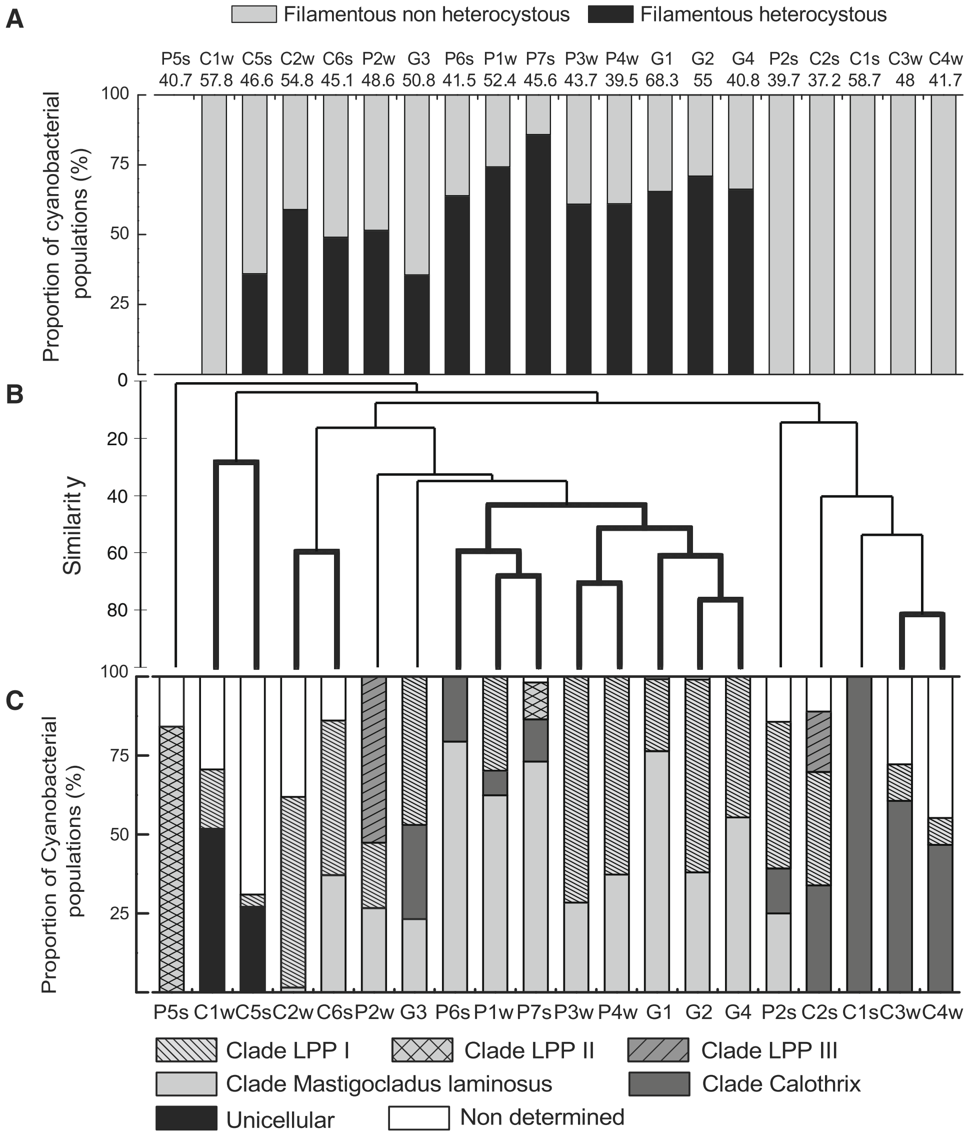

Fig. 3 **a** Proportion of filamentous heterocystous and non-heterocystous cyanobacteria in each sample according to their relative band intensity in 16S rRNA-DGGE fingerprints obtained with general bacterial primers. Temperature (°C) appears below the sampled points. **b** Dendrogram comparing all samples according to the relative band intensities of the different cyanobacterial groups. The *vertical axis* is shown as the Bray–Curtis similarity distances. **c** Relative composition of each cyanobacterial assemblage based on DGGE band intensities with specific cyanobacterial primers. The letters *w* and *s* in sample code indicate winter and summer, respectively

identified, mainly due to sample C5s (Fig. 3c) that showed difficulties in amplification and further sequencing of the extracted bands.

As expected, most bands retrieved belonged to the filamentous cyanobacteria, either HCYA (four bands) or NHCYA (nine bands). The former fit into two different clusters: *Mastigocladus* and *Calothrix* (Fig. 4a). In turn, the NHCYA could be placed in three clusters that we have named LPP-I, LPP-II, and LPP-III (Fig. 4b).

One of the most important bands in all hot springs, CYA22, together with other two bands (CYA27 and CYA46), were affiliated with the genus *Mastigocladus*, a typical thermophylic, branching, heterocyst-containing cyanobacterium of the order Stigonematales. Band CYA22 accounted for the highest band intensity, particularly in Porcelana Geyser and Hot Spring in winter. It was present in low abundance in Cahuelmó and showed intermediate values in Porcelana Hot Spring in summer. Band CYA15 was closely affiliated with *Calothrix* spp., another HCYA (Fig. 4a).

Nine bands were related to cyanobacteria of the filamentous non-heterocystous Order Oscillatoriales. Many of these were members of the thin filamentous genera *Leptolyngbya* and *Oscillatoria*, which lack distinctive morphology and have a poorly resolved taxonomy (Litvaitis 2002). Bands CYA21, CYA20, and CYA28 were widespread among all three hot springs and accounted for large percentages of the total band intensity (over 40 %) in Porcelana Hot Spring in winter and Porcelana Geyser. However, none of them were present in all samples. The other bands corresponded to ill-defined environmental clones from diverse hot spring mats all over the world, and always occurred in low percentages (Figs. 3c, 4b).

At the highest temperature of 68.3 °C at Porcelana Geyser mats, the *Mastigocladus*-like band accounted for 20.6 % of the total band intensity, and two Oscillatoriales-related bands accounted together for 13 %. The temperature at Porcelana Geyser is above the currently accepted upper limit for filamentous cyanobacteria, which is around 55–62 °C (Seckbach 2007). *Mastigocladus* is a genus commonly found in hot environments such as Yellowstone, USA (Miller et al. 2007, 2009), Costa Rica (Finsinger et al. 2008), Italy, Iceland, New Zealand, Russia, and Chile (Miller et al. 2007), among others. As described by Miller et al. (2007), the presence of *Mastigocladus* in such distant places, and the high resemblance of sequences could be related to a recent dispersal event, and with time, new strains or even species might arise in each of these biogeographical islands.

A cluster analysis with all DGGE bands retrieved (including the non taxonomically identified ones) separated Cahuelmó samples from those of Porcelana and Porcelana Geyser with only a few exceptions (Fig. 3b). It appears, therefore, that the cyanobacterial assemblages do not follow the same geographical distribution as the total bacterial assemblages (Fig. 2a). In this case, geographical proximity might be the reason for the closer clustering of Porcelana Geyser and Porcelana Hot Spring. When the dendrogram is compared with the percent composition of each sample in Fig. 3c, it seems that the significant presence of members of the *Mastigocladus* clade determines the clustering together of most Porcelana Hot Spring and all Porcelana Geyser samples. This clade was absent from most Cahuelmó samples. In fact, when this group was removed from the analysis, the similarity index between all samples decreased almost 20 % between Porcelana Hot Spring and geyser, and 6 % between both springs and Cahuelmó. This was already the case with our general bacterial primers analysis. The cluster formed by four Cahuelmó samples and sample P2s from Porcelana Hot Spring seemed to be determined by the massive presence of members of other cyanobacteria, the *Calothrix* clade. Therefore, among the HCYA, the *Mastigocladus* clade predominated in Porcelana Geyser and Hot Spring, while the *Calothrix* clade did so in Cahuelmó, although not in all samples. Thus, different heterocystous cyanobacteria were dominant in different springs. A possible explanation for this finding could be the origin of both genera. While *Calothrix* has both freshwater and marine species and is not reported as a thermophylic genus, *Mastigocladus* is a typical thermophylic genus that has only been reported in freshwater hot springs. As mentioned, Cahuelmó Hot Spring is in close proximity to the fjord waters, and the mats are partially submerged in marine or brackish waters during high tides.

Independently of the primer sets used, *Mastigocladus*-related sequences were detected in 13 samples and were absent in five samples (all from Cahuelmó, three in winter and two in summer). The only discrepancies between both sets of primers were found in samples P2s and C5s. In sample C2w, the relative representation of this cluster was close to 50 % with general bacterial primers but only a very small percent with the cyanobacterial primers. Both samples C5s and C2w had a large proportion of the band intensity unidentified with the cyanobacterial primers, and this could be the reason for the discrepancies. We do not have an explanation for the case of sample P2s.

Order Thermales

The Order Thermales is commonly found in hot environments (Brock 1997). A *Thermus* species was detected for the first time in a hot spring in Yellowstone, USA by Brock and Freeze (1969). The heterotrophic nature of this group places them as consumers of the organic matter

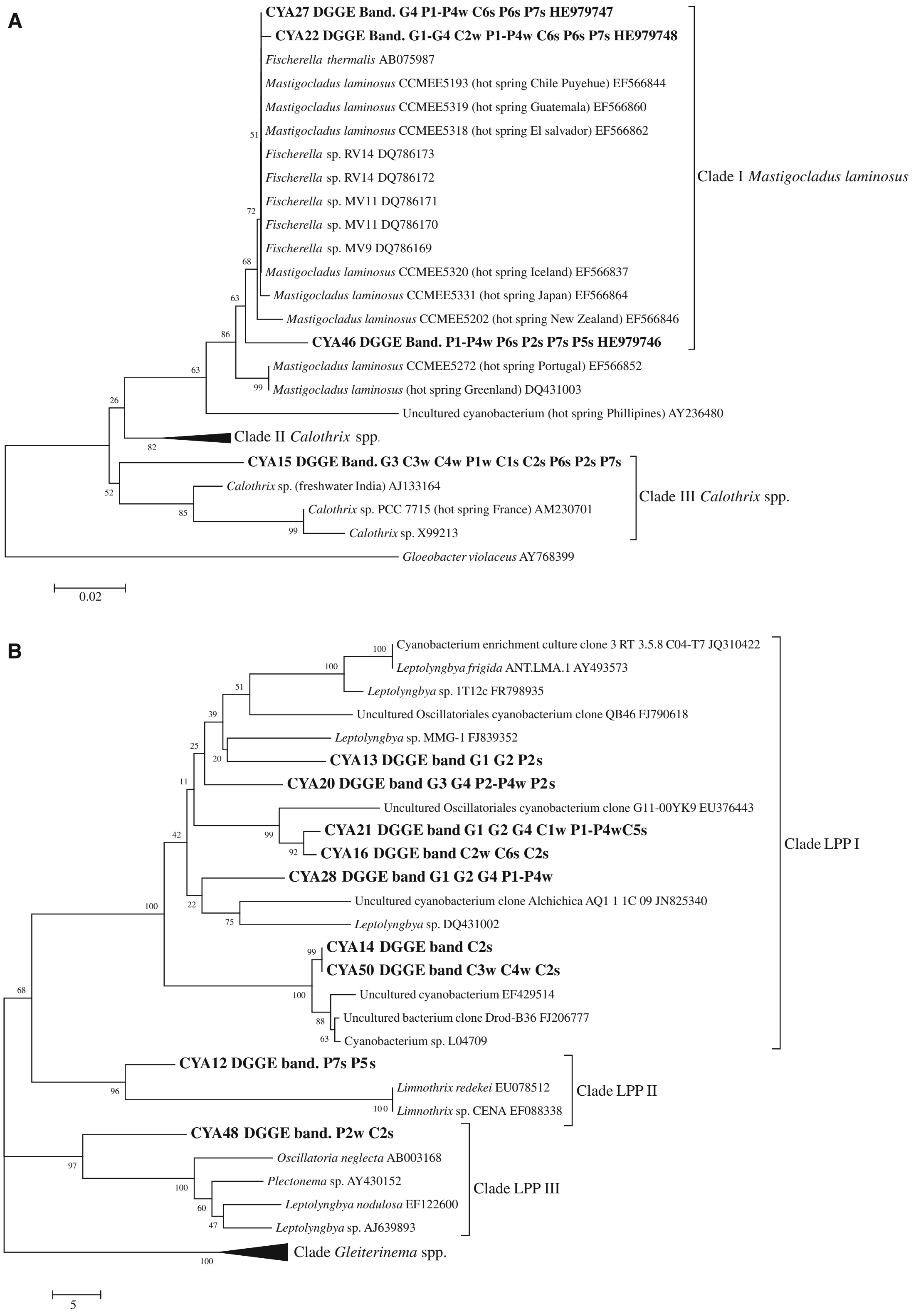

Fig. 4 Phylogenetic reconstruction including the partial 16S rRNA gene sequences obtained by DGGE bands of **a** cyanobacterial populations with heterocysts; **b** cyanobacterial populations without heterocysts, denominated *Leptolyngbya*, *Phormidium,* and *Plectonema* (LPP) group. The 16S rRNA gene sequences used to generate the reconstructions were obtained with specific cyanobacterial primers. Samples where *every band* was present are indicated after the name of the band

produced by Cyanobacteria and other phototrophic, as well as lithotrophic, primary producers in these extreme ecosystems.

This order was well represented in most of our samples, but its contribution varied significantly among sites. Its average contribution to the total band intensity was 20.6 %, ranging between 4 and 53.7 % with one exception: no Thermales sequences could be retrieved from sample P5s. This sample was already mentioned in the section on Cyanobacteria as it had a large percent of undetermined bands (Fig. 2b). The presence of Thermales decreased from 24.1 and 30.1 % in Cahuelmó (in winter and summer, respectively) to 19.8 % in Porcelana Geyser, and down to 12 % in Porcelana in both winter and summer.

Considering each sample individually, the percent contribution of Thermales ranged between 4 and 26 % throughout the temperature gradient (except the above mentioned sample P5 s). Two points with temperatures between 57 and 59 °C showed the highest contributions around 52 % (Fig. 5b). If this can be taken as an indication of the temperature optima of the Thermales present in these springs, it would coincide with the optimum found for members of the genus *Meiothermus* (50–65 °C in different species, Nobre et al. 1996). The genus *Meiothermus* was formerly included in the genus *Thermus* (Nobre et al. 1996), but the former has lower temperature optima, and the two genera use different niches (Chen et al. 2002).

The lower percentage of Thermales found at the highest temperature (16 % at 68.3 °C) suggests that no representatives of the genus *Thermus* were present (with optimal temperatures around 70–75 °C, Nobre et al. 1996). In fact, the two DGGE bands that could be assigned to the Thermales (bands BG2 and BG16) clustered with the genus *Meiothermus* (Fig. 6a). Their closest relatives were *Meiothermus ruber* and an environmental sequence recovered from a hot spring in Thailand (Portillo et al. 2009). Band BG16 was absent from samples with temperatures above 50 °C (Fig. 5b). This band was absent from Porcelana Geyser, increased with decreasing temperature in Cahuelmó (up to 30 % in winter, and to almost 60 % in summer), and was more represented in Porcelana not only at temperatures around 40 °C, but also at 45 °C in sample P7s (Fig. 6b). Band BG2, on the other hand, thrived throughout the temperature gradient, but reached its highest relative abundance above 55 °C. This was absent from Porcelana in summer and was less important than BG16 in winter, and its importance decreased with decreasing temperature in Cahuelmó. Thus, it appears that some other environmental parameter besides temperature was influencing the distribution of these two possible ecotypes.

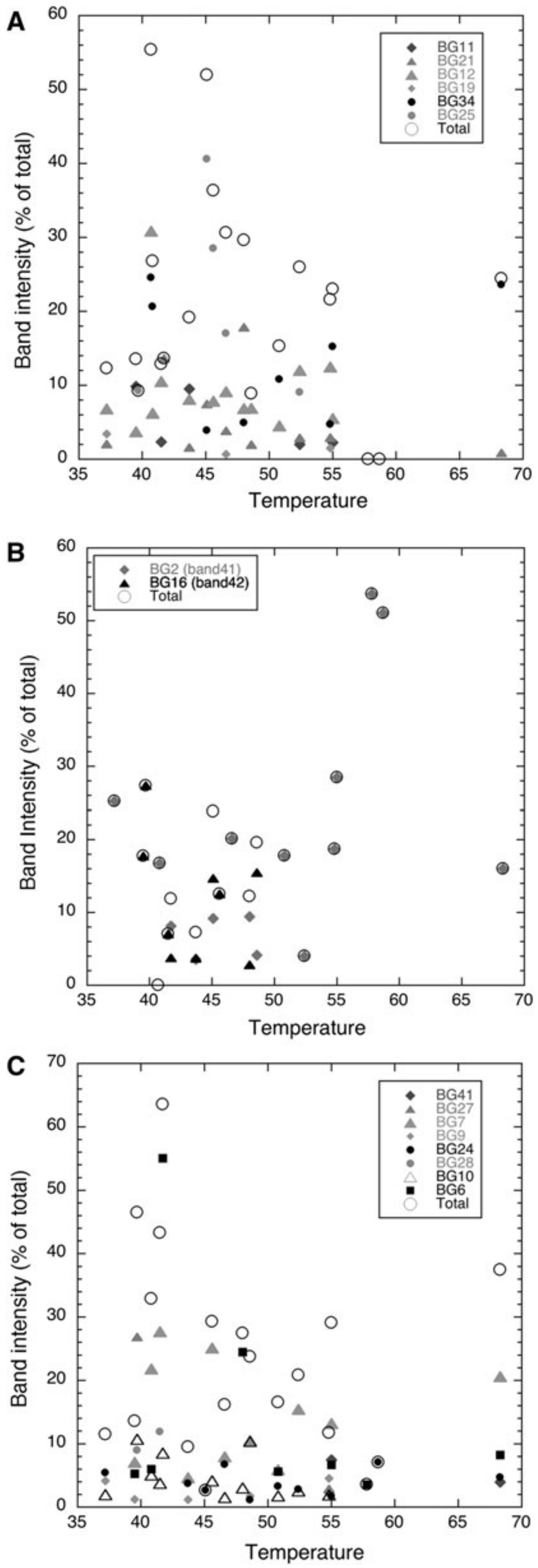

Fig. 5 **a** Relative abundance of the cyanobacterial affiliated bands with respect to temperature. **b** Relative abundance of the Thermales affiliated bands with respect to temperature. **c** Relative abundance of the Bacteroidetes affiliated bands with respect to temperature

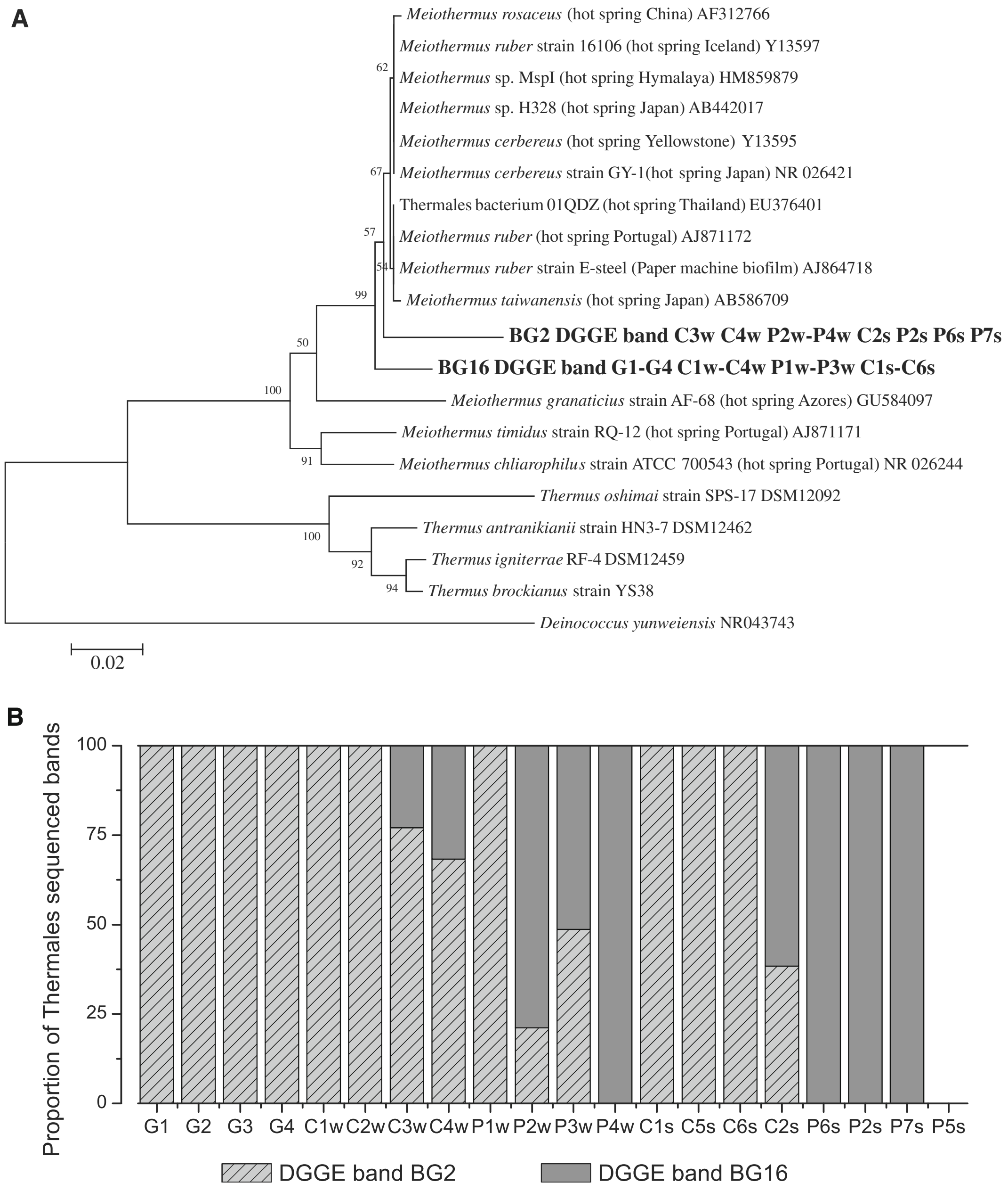

Fig. 6 **a** Thermales phylogenetic reconstruction including sequences retrieved from partial 16S rRNA sequences obtained from DGGE bands using universal bacterial primers. Each sequence includes the sites where they were found. **b** Percent of relative contributions in each sample of the two Thermales bands identified in the DGGE analysis using the universal bacterial primers

Phylum Bacteroidetes

The class Bacteroidetes is a heterotrophic bacterial group not associated specifically with hot springs. Nevertheless, in the past decade, some studies have reported Bacteroidetes as an important group in hot springs from Thailand (Kanokratana et al. 2004; Portillo et al. 2009). The high diversity of species in this group also includes a wide variety of heterotrophic metabolisms, representing potentially important consumers of the organic matter produced by Cyanobacteria and other autotrophic organisms such as the green non-sulfur bacteria from the genus *Chloroflexus*, also detected in the study.

Class Bacteroidetes was a very well represented group in most of our samples, accounting for 22.4 % of the total band intensity in Porcelana Geyser, 15.8–22.7 % in Cahuelmó (winter and summer, respectively) and 16.9–28.5 % in Porcelana (winter and summer, respectively). No relationship with temperature was apparent for any of the clades retrieved (Fig. 5c). This group of bacteria

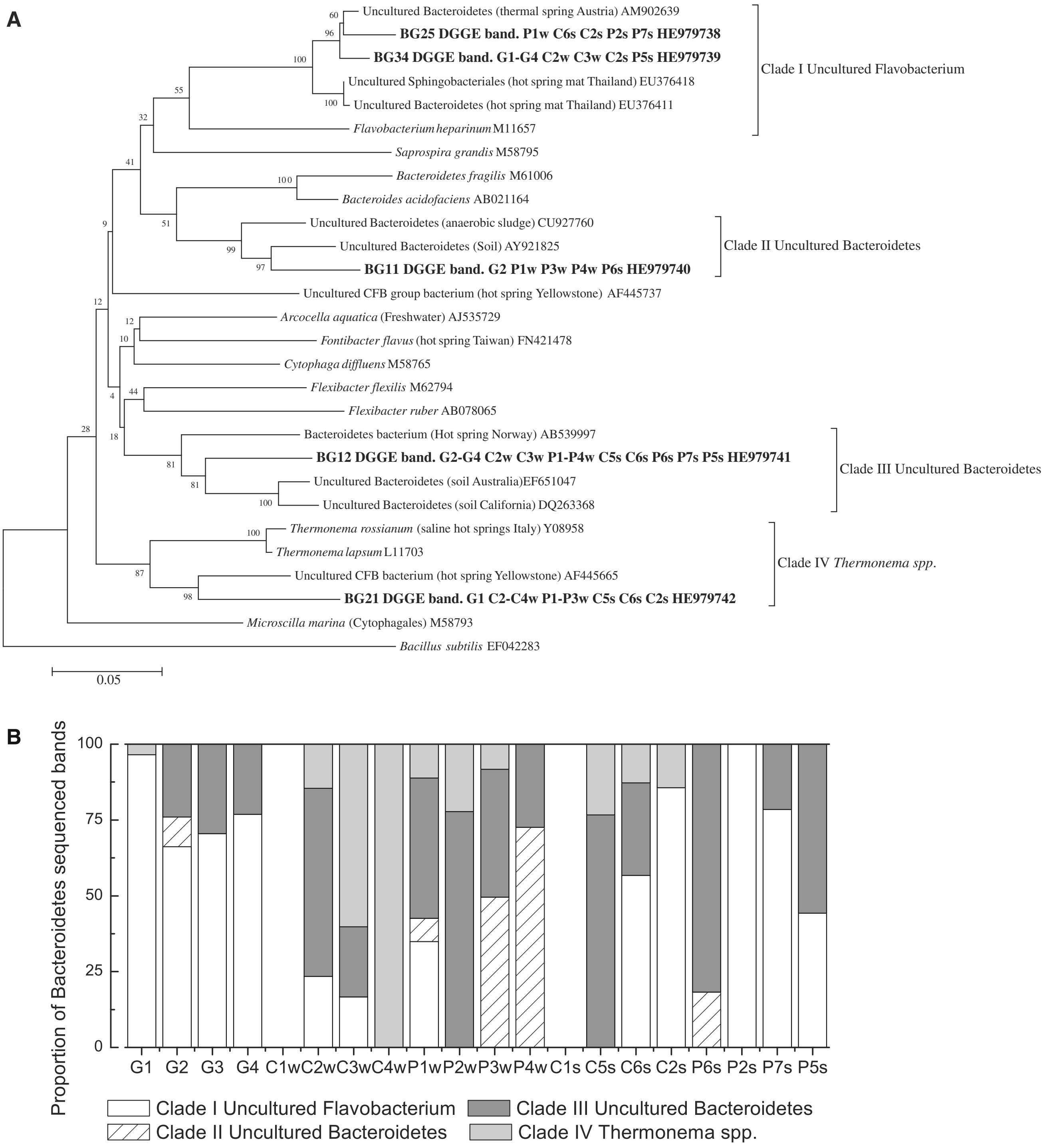

Fig. 7 **a** Bacteroidetes phylogenetic reconstruction including partial 16S rRNA sequences retrieved from DGGE bands using universal bacterial primers. Each sequence includes the sites where they were found. **b** Percent of relative contributions of the four clades of Bacteroidetes retrieved in the different samples

was absent only from the two hottest points, around 58 °C, in Cahuelmó (Fig. 2b). Since Bacteroidetes were present at higher temperatures in Porcelana Geyser, clearly high temperature was not the reason for this absence in Cahuelmó. In the other Cahuelmó samples the contribution of Bacteroidetes ranged between 9 and 55 %. In both Porcelana and Cahuelmó, their contribution seemed to increase with decreasing temperature in summer, but not in winter. It is worth noting that the temperature range was very similar in both seasons (Table 1).

Five discrete bands provided good sequences related to Bacteroidetes and were incorporated into the phylogenetic reconstruction of this class (Fig. 7a). They appeared in four different clusters, named I to IV for the present discussion. The relative band intensity contributions to the total Bacteroidetes can be seen in Fig. 7b. Clade I was the most abundant in Porcelana Geyser and in the summer samples from both Cahuelmó and Porcelana Hot Spring (Fig. 7a). The two bands in this cluster were related to sequences from hot springs in Thailand as their closest relatives (Portillo et al. 2009). Clade II was related to Bacteroidetes sequences from soil and was detected in low-temperature samples from Porcelana Hot Spring, and in one sample from Porcelana Geyser. Clade III, on the other hand, was significant in almost all samples. It was related to the Bacteroidetes bacterium sequence AB539997, with over 95 % of similarity. Clade IV was represented by only one band associated with the genus *Thermonema* and its closest relative was a sequence from a saline hot spring in Naples, Italy (Tenreiro et al. 1997) (Fig. 7a). This population was well represented in Cahuelmó, especially in winter, and it was present also in Porcelana (winter only). The *Thermonema* isolate sequence reported by Tenreiro et al. (1997) was slightly halophilic, which could explain its presence in Cahuelmó, as it is the only hot spring studied subjected to seawater influence.

Other bacterial groups

Acidobacteria, Green Non-sulfur Bacteria, and Beta-, Gamma-, and Epsilon Proteobacteria, were detected in small relative abundances and not in all the samples investigated in this study. A high diversity of Proteobacteria and Acidobacteria members have been also reported in hot environments from volcanic areas in the Canary Islands (Portillo and Gonzalez 2008), and in western Thailand (Portillo et al. 2009). Phototrophic green non-sulfur bacteria *Chloroflexi* were also well represented in tropical hot spring from the island of Luzon, in the Philippines (Lacap et al. 2007). The metabolisms found among these bacterial groups in hot springs are diverse, from autotrophic photosynthetic *Chloroflexi* (Lacap et al. 2007) and chemolithotrophic epsilon-proteobacteria (Takai et al. 2005) to anaerobic heterotrophs, which have been shown to be abundant in hot springs well above the temperature limit for photosynthesis (Kristjansson et al. 1985). The three hot springs in this study have different surrounding environmental conditions, and the diversity of organic substrates must be causing differences in the development of the heterotrophic fraction of microbial communities, as shown in our fingerprinting analyses (Figs. 2b, 8).

Altogether, the overall similarity found in bacterial communities from Porcelana Hot Spring and Porcelana Geyser microbial mats and the differences of both with Cahuelmó (Fig. 8) suggest the influence of geographical distance as well as differences in the geological substrate in these hot spring environments. Additionally, the influence of seawater in Cahuelmó hot sprint, although with low salt concentration (3 %), might also affect bacterial diversity, for example, in the case of the slightly halophilic *Thermonema* spp.

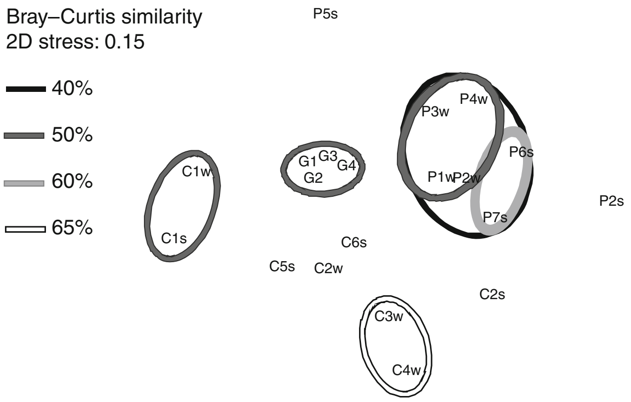

Fig. 8 Multidimensional scale analysis of all samples by Bray–Curtis similarity distances. Similarity distances between samples are shown in *circles*

Small seasonal variations in bacterial communities were detected in Porcelana Hot Spring (Fig. 8). Porcelana Hot Spring samples were clustered with over 40 % similarity in both seasons except for samples P5s and P2s, both from summer, although this hot spring showed a variation of 7 °C in the hottest points (P1w and P7s) between winter and summer. On the other hand, Cahuelmó Hot Spring did not show a temperature variation between winter and summer. No clear similarities were observed among samples in this hot spring, except for samples C1s and C1w, with the same temperature, which showed over 50 % similarity. As mentioned before, Cahuelmó Hot Spring is the only hot spring of the three that experience to some extent human intervention, except for points C1s and C1w (source of the spring) where the temperature is so high that remains free of attention by tourists. Our results suggest that the temperature at these hot springs is differently affected by seasons. According to the results retrieved, the temperature would be the main—but maybe not the only—environmental factor influencing the bacterial community structure and composition in the microbial mats present in these ecosystems.

Also, the fingerprint similarity between Porcelana Hot Spring and Geyser and the dissimilarity of both with Cahuelmó Hot Spring may be due to geographical distance and rock-substrate differences.

As the economical value of these hot springs is being assessed in terms of ability to attract tourists, it is now very important to document the biodiversity of these biologically unknown environments and to recognize other potential values such as their genetic resources for the developing field of biotechnology. Thus, a more sustainable management can be achieved in the coming years for these hot springs in Northern Patagonia.

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