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Phylogenetic diversity and temporal variation in the *Spirochaeta* populations from two Mediterranean microbial mats

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Summary. Spirochetes are among the bacterial groups often observed in hydrogen-sulfide-rich layers of coastal microbial mats. However, relatively few spirochetes from these microbial mats have been described and characterized. We used 16S rDNA phylogenetic analysis to investigate the spirochetal diversity of microbial mats from two locations in the western Mediterranean (Ebro Delta, Spain, and Camargue, France). Samples from each location were monitored in the spring and winter over a period of 1 to 2 years. In the sequence analysis of 332 clones derived from samples of both locations, 42 novel phylotypes of not-yet-cultivated spirochetes belonging to the genus *Spirochaeta* were detected. None of the phylotypes were identified as known culturable species of *Spirochaeta* or previously identified phylotypes cloned from other hypersaline microbial mat such as Guerrero Negro, Mexico. Eight of the phylotypes were common to Ebro and Camargue mats, and two (IF058 and LL066) were present both in spring and winter. Some phylotypes appeared to show seasonal variation, i.e., they were found only in the spring, but not in the winter. Ebro and Camargue phylotypes, like phylotypes from Guerrero Negro, grouped according to the vertical gradient of oxygen and sulfide in the mat. Some phylotypes, such as LH073, IE028, LH042, or LG013 were harbored in low H_2S or H_2S–O_2 interface zone. In contrast, major phylotypes were detected in deeper layers and they were likely strict anaerobes and high tolerant to H_2S. The presence of spirochetes in differently located microbial mats suggests that they constitute very diverse and stable populations involved in a well-integrated metabolic symbiosis (i.e., permanent physiological cooperation) with other guild populations in the mats, where they maintain a coordinated functional and stable community [**Int Microbiol** 2008; 11(4):267-274]

Key words: *Spirochaeta* species · bacterial phylogenetic diversity · temporal variation · microbial mats · symbiosis

Introduction

Biofilms are bacterial surface-associated communities attached to solid substrata, growing into and embedded in a polymeric matrix produced by bacteria. Microbial mats can be considered multilayered complex biofilms [17,24,32]. Microbial mats are stratified microbial ecosystems. Laminae form as a results of light extinction with depth and physicochemical microgradients due to the metabolism of different bacterial populations [17]. Lamination reflects a complex structure organized at the millimiter-depth and the micrometer-depth spatial scale [17,41]. Such ecosystems contain members of all trophic levels and are characterized by cyclic seasonal fluctuations of flooding and desiccation, and by diel fluctuations in the concentrations of oxygen, sulfide, and other chemical nutrients [8,23]. Microorganisms rapidly respond to changes in various physicochemical gradients, loc-

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ating themselves according to the most favorable environmental conditions. This behavior is likely to govern the vertical species stratification that results from the active migration of motile cells in response to the shifting gradients of electron donors and/or acceptors observed within microbial mats [18]. Microbial mats exemplify functionally integrated, self sustaining, laminated microbial consortia [9,17,31], showing remarkably high degrees of biodiversity compressed into a few millimeters. In 2006, molecular analysis of Guerrero Negro microbial mats revealed 42 phyla (including 15 novel candidate phyla) [26]. In comparison, soil can contain more than 20 bacterial phyla [11]; approximately 12 phyla are present in the Sargasso Sea [36]; the adult human gastrointestinal tract, with 8 phyla [2], and healthy human skin, also with 8 phyla [15], are the environments with the lowest number of phyla present, although they appear to be highly diverse at the strain and species level.

Free-living spirochetes represent one of the bacterial groups often observed in hydrogen-sulfide-rich layers from microbial mats habitats and deep-sea vents [1,6,21,23], where spirochetes may constitute a stable population. It has been observed the cosmopolitan distribution of *Spirosymplokos deltaeiberi*. This species was first described in Ebro mats [16,28], and lately in samples from Sippewissett salt marsh at Woods Hole, Massachusetts, USA, and in microbial mats at North Pond, Laguna Figueroa, Baja California Norte, Mexico. The identity of these spirochetes was confirmed by electron microscopy [29]. Spirochetes represent a group of helical, motile bacteria that are widely distributed in nature, and they constitute a monophyletic phylum characterized phenotypically by a cellular ultrastructure and their manner of motility that are unique among the domain *Bacteria* [32].

The primary purpose of this study was to use culture-independent molecular methods to determine and compare the *Spirochaeta* species diversity in samples from two Mediterranean microbial mats: Ebro Delta (on the northeastern coast of Spain), and Camargue (on the south coast of France). These data were compared with certain cultivable species (15 cultivable species of *Spirochaeta* are considered to be presently known [25]), or previously reported phylotypes. The major difference between the two geographical sites is their salinity. Ebro Delta salinity ranges from 40 to 70% [30], whereas the salinity of Camargue is higher, ranging from 60 to 140% [40]. Salinity has been reported to influence microbial mat composition and to be reflected in the diversity of the entire prokaryotic community [1,41]; hence, it may also control spirochetal diversity. The secondary purpose was to begin to estimate the seasonal variation of *Spirochaeta* diversity by monitoring samples taken over the course of 1 to 2 years.

Materials and methods

Sample collection. Samples were collected from laminated, intertidal microbial mats from the western Mediterranean Sea: Alfacs peninsula, Ebro Delta, northeastern Spain (0° 35′ to 0° 56′ E; 40° 33′ to 40° 47′ N), and Camargue, Rhône Delta, southern France (4° 11′ to 4° 57′ E; 43° 40′ to 44° 40′ N) (Figs. 1 and 2). Mat samples were collected in cores (1 cm × 3 cm) that were sliced horizontally in 2-mm increments (from the top to a depth of 6 mm) at 12.00 h. Collection from the Ebro Delta took place in May 2001, November 2002, and May 2003; sampling from Carmargue took place in April 2002, November 2002, and April 2003.

DNA extraction. DNA was extracted from 2–6 mm depth slices. Pieces of microbial mat of approximately 1 mm^3 from each slice were suspended in 100 µl TE buffer in 2.0 ml vials with a capful of 0.1 mm glass beads. The mixture was then homogenized for 1 min in a Minibeadbeater-8 (Biospec Products, Bartlesville, OK, USA) and centrifuged at high speed for 2 min. With careful avoidance of the transfer of beads, 50 µl of each sample ware pipetted into sterile 0.5 ml Eppendorf tubes. DNA was extracted with a phenol-chloroform mixture and precipitated in the cold with 95% ethanol. Aliquots were resuspended in 20 µl TE pH 7.5 and stored at –20°C to preserve the DNA until needed.

PCR and cloning. A spirochetal selective reverse primer, 5′-GTTACGACTTCACCCYCCT-3′, was used with a universal forward primer, 5′-GAGTTTGATYMTGGCTCAG-3′, to selectively amplify spirochetal 16S rRNA genes from environmental samples [10]. PCR amplification was performed in 50 µl final volume of reaction mix: 1 µl of the DNA template, 20 pmol of each primer, 40 nmol of dNTPs, 1.5 mM $MgCl_2$ and 1 U of Taq platinum polymerase (Invitrogen, San Diego, CA, USA). Samples were preheated at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 45 s, annealing at 60°C for 45 s, and elongation at 72°C for 1.5 min, and finally an elongation step at 72°C for 15 min. The results of PCR amplification were examined by electrophoresis in a 1% agarose gel. DNA was stained with ethidium bromide and visualized under short-wavelength UV light. PCR products were purified by a QIAquick Gel extraction kit (Qiagen, Valencia, CA, USA) and cloned using the TOPO TA cloning kit (Invitrogen), according to the manufacturer's recommendations.

16S rRNA sequencing. Purified PCR products were sequenced using an ABI prism cycle-sequencing kit (BigDye® Terminator Cycle Sequencing kit with AmpliTaq DNA polymerase FS, Perkin-Elmer, Boston, MA, USA). The primers used for 16S rRNA sequencing were as previously described [33]. Half dye or quarter dye chemistry was used with 3.2 mM primers, 3 µl PCR product in a final volume of 20 µl. Cycle sequencing was performed using an ABI 9700 with 25 cycles of denaturation at 96°C for 10 s, and annealing and extension at 60°C for 4 min. Sequencing reactions were run on an ABI 3100 DNA sequencer.

Data analysis. Over 300 clones with the correct size insert of approximately 1,500 bases were analyzed. About 500 bases were first sequenced to determine approximate phylogenetic position. Then, full sequences were obtained for representatives of novel *Spirochaeta* phylotypes. The 16S rRNA sequences were compared to known sequences in GenBank with the advanced gapped BLAST (basic local alignment search tool) algorithm. Phylogenetic analyses were performed using MEGA version 2.1. The dendrogram was constructed using the neighbor-joining algorithm and the Kimura 2-parameter distance estimation method. Two hundred bootstrap trees were generated, and bootstrap confidence levels were determined using the MEGA 2.1 program. Chimeric sequences were identified by using the Chimera check program in the Ribosomal Database Project II [7], by treeing analysis, or base signature analysis.

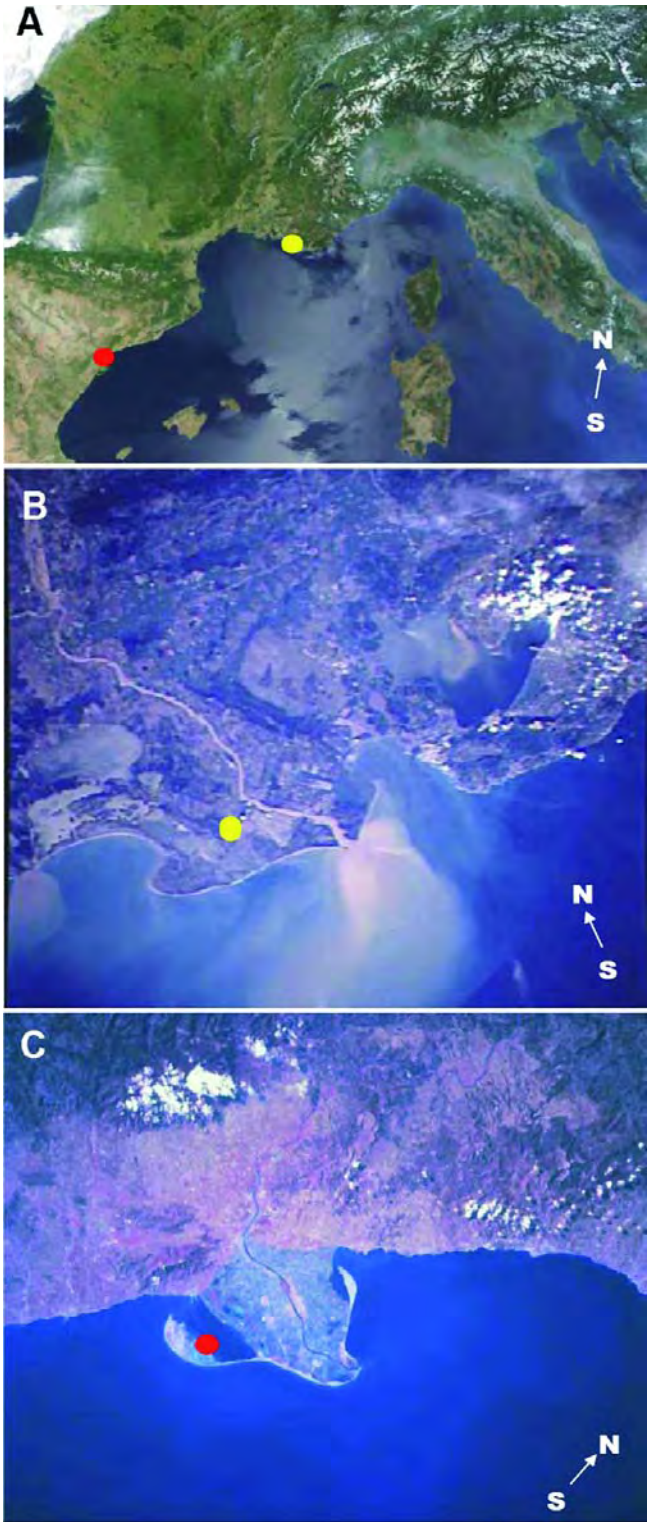

Fig. 1. (**A**) Northwestern Mediterranean area. (**B**) Sampling locations in Camargue. (**C**) Sampling locations in Ebro Delta.

Nucleotide sequence accession numbers. The complete 16S rRNA gene sequences of clones representing novel phylotypes defined in this study and published sequences are available for electronic retrieval from the EMBL, GenBank, and DDBJ nucleotide sequence databases.

Fig. 2. Macroscopical view of the Camargue microbial mat sampled in the present study. Bar = 25 cm.

Results

A total of 332 clones were sequenced in order to establish the diversity of spirochetes in two microbial mats located in the western Mediterranean Sea. Temporal variation of spirochete diversity at each location was also examined. Forty-two new *Spirochaeta* phylotypes were detected in these mats (Fig. 3). In Ebro Delta samples there were 33 phylotypes, of which 25 were unique to this location. In Camargue samples, there were 17 phylotypes, of which 9 were unique. Eight phylotypes were common to both samples.

None of the phylotypes were identified as known cultivable species of *Spirochaeta*. Phylotype IE071 grouped with *Spirochaeta bajacaliforniensis* and *S. smaragdinae*, both of which are obligate anaerobes. LH073 clustered with *S. alkalica*, *S. africana* and *S. halophila*. The first two species are aerotolerant anaerobes, halophilic, and were isolated from sediments of alkaline lakes; *S. halophila* is a facultative anaerobe isolated from a high-salinity pond.

Temporal variation was observed with the spirochetal phylotypes IE029, IE044, IE090, IE070, which were detected only in springtime samplings, but seem to disappear in the cold season. Phylotypes IF058 and LL066 were present both in spring and winter, and in both Ebro and Camargue mats.

None of the Ebro and Camargue phylotypes were identified as previously phylotypes reported from other hypersaline microbial mats such as Guerrero Negro (Fig. 4). Although we did not study spirochetal diversity at different depths in Ebro and Camargue mats, when we compared them with spirochetes from Guerrero Negro we observed two

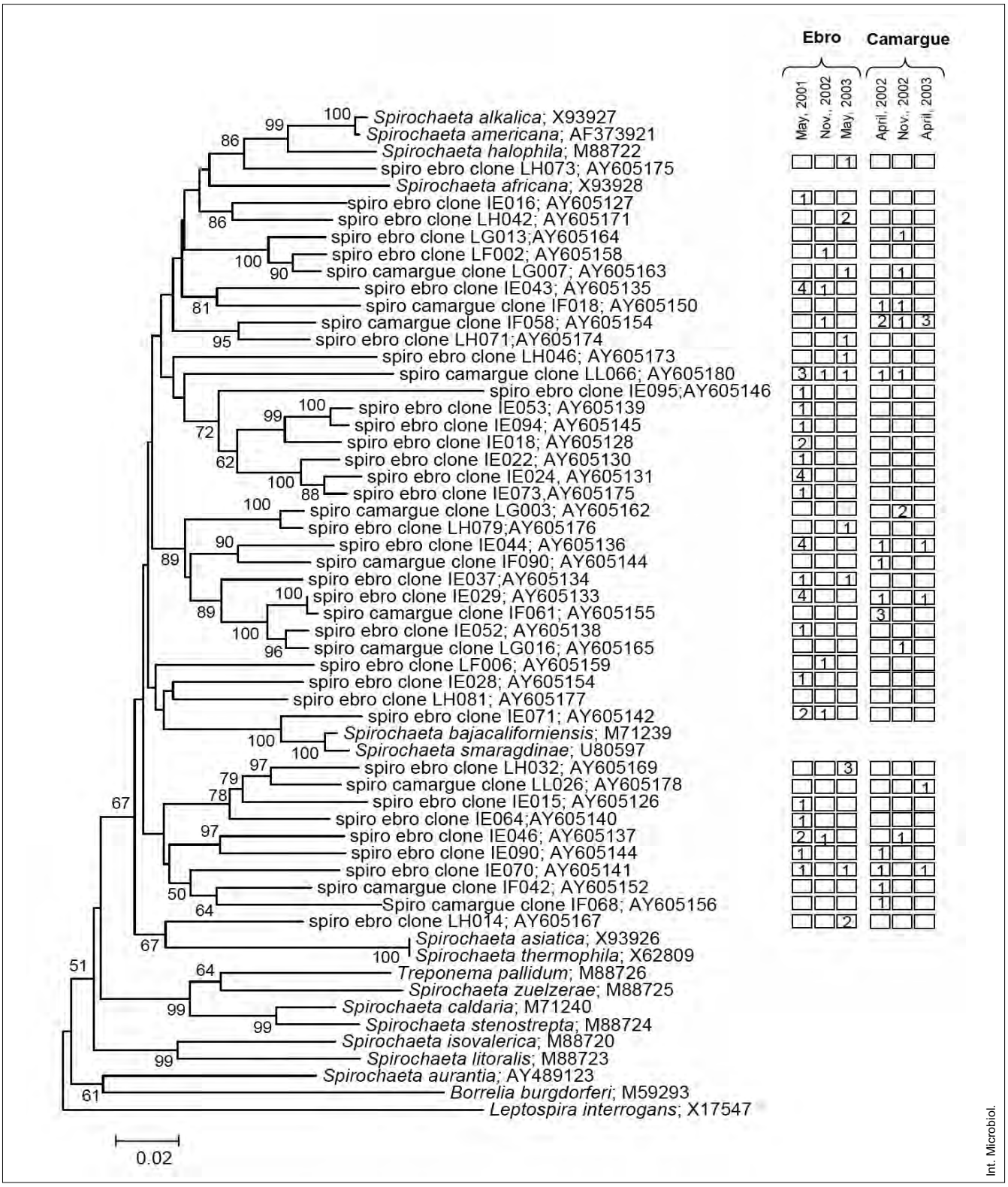

Fig. 3. Spirochetal phylotypes of the genus *Spirochaeta* detected from the Ebro Delta and Camargue mats. The information presented includes spirochetal species or phylotype clone ID and sequence accession number. Novel phylotypes are defined as those taxa that are <98.5 similar in sequence comparisons to their closest relatives. Columns to the right indicate the presence of a phylotype in each location at different sampling dates. Numbers in boxes represent the number of clones in each nucleotide sequence. One-thousand bootstrap trees were generated,and bootstrap confidence levels as percentages (only values over 50%) are shown at tree nodes.

major groups according to their location in the oxic or the anoxic zone. (i) Phylotypes LH073, LH042, and IE016 clustered with phylotypes detected at 0–2 mm depth in Guerrero Negro mats sampled during the day. These phylotypes also grouped with aerotolerant or facultative anaerobe cultivable *Spirochaeta* (Fig. 3). (ii) Most of the remaining Ebro and Camargue phylotypes clustered with the Guerrero Negro phylotypes present at >2 mm depth (collected both during the day or night). These phylotypes are likely to be obligate anaerobes.

Discussion

Although spirochetal populations in microbial mats seem to represent only 1% to 4% of the total population, spirochetal diversity at the species taxonomic level was remarkably higher in microbial mats of the Ebro and Camargue than in Guerrero Negro mats [26]. Ley et al. [26] have described microbial diversity within the Guerrero Negro mats divided vertically on a millimetric scale. Most of the spring Ebro and Camargue spirochete phylotypes in the present study grouped with October samples from Guerrero Negro, in which the temperature and irradiance during the autumn and the spring might be similar.

It has been suggested that diversity of competing or related phylotypes coexisting may be associated with both spatial and temporal segregation in spatially heterogeneous environments that challenge the metabolic and regulatory repertoire of the indigenous populations [19,22,39]. Phylotypes may complement each other in space, so spatial gradients allow occupation by more diverse species. Diversity may act as insurance for ecosystem functioning against temporal environmental change, with functional compensations among phylotypes as showed in both seasonally and diel fluctuations in microbial mats [6,26,37,38].

Five *Spirochaeta* species may share physiological characteristics wtih our phylotypes (i.e., IE071 and LH073) because the environmental conditions are similar [14,25,27]. *Spirochaeta bajacaliforniensis* is a marine spirochete isolated from Laguna Figueroa mats (Baja California, Mexico) [14]. *Spirochaeta smaragdinae* was isolated from an oil field (Congo, Africa); it grows optimally with a sodium chloride concentration of 5%, and reduces thiosulfate and sulfur to H_2S [27]. There were no close relationships between our phylotypes and *S. asiatica*, *S. thermophila*, *S. zuelzerae*, *S. caldaria*, *S. stenostrepta*, and *S. aurantia*. Indeed, the species *S. caldaria*, *S. stenostrepta* and *S. zuelzerae*, although free living spirochetes, grouped within the genus *Treponema* on the basis of 16S rRNA sequence analysis [32]. (See Leschine et al. [25] for a review.)

Temperatures change dramatically between summer and winter seasons. During winter, ambient temperatures are lower and daily temperature variations (day-night) are less pronounced than in summer, so less pronounced daily temperature variation in winter may have favored the adaptation of the microbial population to lower temperatures, possibly explaining the seasonal distribution of several phylotypes, such as IE029, IE044, IE090, and IE070. The adaptation of populations to different temperatures may help to provide homeostasis within a mat community; i.e., temperature-adapted populations could stabilize community functions against flow-related or seasonal variation in temperature [4,39,40]. Fluctuation or seasonal variation in population distribution of 16S rRNA phylotypes has been observed in a hot spring microbial mat [4,13], diazotrophic bacteria in Chesapeake Bay [34], or in temperate stream habitats [20] that have distinct nonrandom spatial and seasonal distribution reflecting either specific physical processes or adaptations to different environmental niches.

Diel fluctuations in the concentrations of oxygen and sulfide shape the chemical environment and provide daily contrasting microenvironments that are separated on a scale of a few millimeters. During the day, the redox cline may occur at a depth of approximately 1–2 mm. Oxygen concentration within the mats we studied increased during the day until the afternoon, and then decreased with decreasing irradiance. When oxygenic photosynthesis ceases at night, the upper layers of the mat become highly reduced and sulfidic [26,30,38, 40]. Microorganisms orient along microscale chemical gradients (i.e., O_2, pH, E_h) to meet and optimize the biogeochemical processes (C, N and S cycling), which are essential for survival, growth and maintenance of genetic diversity, needed to sustain a functional system. Phylotypes LH073, LH042 and IE016 may be aerobic, aerotolerant or facultative anaerobic; they may be present in the upper layers and constitute important population in the sharp transition between the oxic and the sulfidic zone, that is characterized by a narrow zone of overlapping O_2 and sulfide gradients.

Trophic interactions play a major role in tying a microbial community together. The functional grouping within the community depends on the quality of substrates and products. This type of interdependence is examplified by an anaerobic community operating as a network of hydrolytic to fermenting primary anaerobes, then to syntrophic bacteria and to homoacetocetic, methanogenic, or sulfidogenic secondary anaerobes. In diverse anoxic environments, spirochetes occupy an intermediate trophic level between the hydrolytic bacteria and these secondary anaerobes; this is because the main compounds produced by spirochete are acetate, H_2, and CO_2, which are normally consumed by sul-

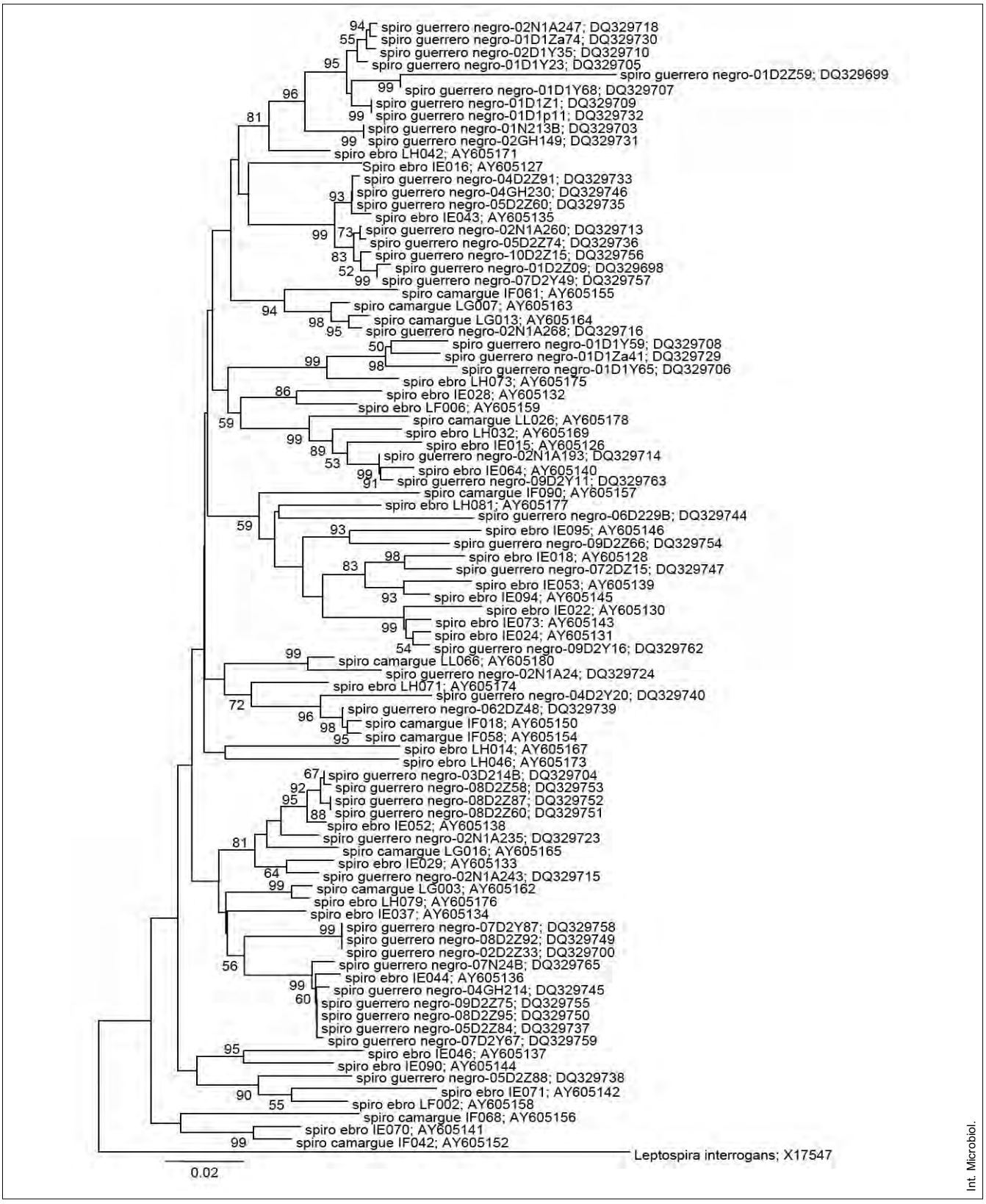

Fig. 4. *Spirochaeta* phylotypes detected from Ebro Delta and Camargue mats compared with *Spirochaeta* phylotypes from Guerrero Negro mats. Phylotypes from Guerrero Negro mats were named according to the layer they were taken from (01 to 10), the time of collection (D: day, N: night), and the month of collection (1: June; 2: October).

fate-reducing bacteria and methanogens [3,5,12]. Spirochetes may constitute a ubiquitous component of microbial mats, playing a significant functional role in the community by supplying carbon sources and electron donors to the other populations. *Spirochaeta* phylotypes persist in anoxic-microoxic sediments and compete effectively with other heterotrophic organisms

This study is a firm first-step toward estimating the diversity of spirochetes in microbial mats. Additional 16S rDNA sequencing needs to be done to establish the spirochetal diversity according to depth in the mat. Once achieved, 16S rRNA-based checkerboard DNA-DNA hybridization assays or microarrays can be used to more accurately determine the bacterial populations' distribution and function within microbial mats.

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