Systematic and Applied Microbiology 41 (2018) 131–138

Contents lists available at ScienceDirect

Systematic and Applied Microbiology

journal homepage: www.elsevier.de/syapm

Vibrio communities in scleractinian corals differ according to health status and geographic location in the Mediterranean Sea

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ARTICLE INFO

Article history:
Received 1 August 2017
Received in revised form 31 October 2017
Accepted 8 November 2017

Keywords:
Vibrio
Oculina
Cladocora
Bleaching
Mediterranean

ABSTRACT

The increase in seawater temperature associated with global warming is a significant threat to coral health and is linked to increasing mass mortality events and *Vibrio*-related coral diseases. In the Mediterranean Sea, the endemic *Cladocora caespitosa* and the invasive species *Oculina patagonica* are the main scleractinian corals affected by mass mortalities. In this study, culturable *Vibrio* spp. assemblages associated with healthy and unhealthy colonies of these two shallow coral species were characterized to assess the presence of *Vibrio* pathogens in tissue necrosis. *Vibrio* communities associated with *O. patagonica* and *C. caespitosa* showed geographical differences, although these became more homogeneous in unhealthy specimens of both species. Furthermore, the number of recovered *Vibrio* specimens was more than five times higher in unhealthy than in healthy corals. Within these culturable vibrios, the known pathogens *Vibrio mediterranei* and *Vibrio coralliilyticus* were present in unhealthy colonies of both coral species in the two localities, suggesting that they could play a role in the health status of *C. caespitosa* and thus act as generalist pathogens in Mediterranean corals. Nonetheless, a clonal type of *V. coralliilyticus* detected in *C. caespitosa* was not associated with disease signs, suggesting that this species could encompass assemblages with different levels of virulence.

Introduction

Vibrio species are heterotrophic bacteria that are geographically spread in marine environments throughout the world. They have often been associated with diseases of humans and marine animals such as shellfish, fish and corals [reviewed in reference 28]. *Vibrio* species are strongly thermodependent and hence their occurrence is more frequent in warm waters [46] and in temperate regions, where their abundances increase during warm seasons [41]. Thus, global warming could promote their proliferation and any potential disease outbreaks associated with *Vibrio* pathogens [2,13,42,43], particularly in temperate regions where the relative increase in seawater temperature seems to be higher than in tropical areas [39]. This effect is especially dramatic in the Mediterranean [22] where the average temperature rise has been approximately 0.04 °C/year over the last three decades [8]. This warming trend is correlated with an increase in mass mortality events of marine life [11].

After the last Mediterranean mass mortality events of sessile invertebrates in 1999, 2003 and 2015 [5,6,35], interest in the potential role of pathogenic microorganisms in such episodes has grown [11]. However, the relationship between pathogens and disease outbreaks has been demonstrated only in a few cases. For example, *Vibrio coralliilyticus* was linked to the disease of the purple gorgonian *Paramuricea clavata* [4,3], and the infection was shown to be regulated by seawater temperatures [46]. More recently, experimental infection in aquaria confirmed the involvement of *Vibrio mediterranei* and *V. coralliilyticus* in *Oculina patagonica* tissue damage [36]. Furthermore, these two *Vibrio* pathogens have been detected by MALDI-TOF in gorgonians, sponges and corals that showed tissue necrosis during a mass mortality event observed in the summer of 2015 in the Western Mediterranean Sea [35].

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https://doi.org/10.1016/j.syapm.2017.11.007

Vibrios are part of the normal microbiota of different coral species and represent more than 40% of the bacteria isolated from corals [30]. Some authors [34] have suggested that vibrios could play an important function in coral health status, although their role is unclear and requires further research. Chimetto et al. [6] demonstrated that some *Vibrio* species isolated from corals could form a mutualistic relationship with the host by fixing nitrogen in the coral holobiont, whereas others are putative agents of coral diseases. Therefore, knowledge of the normal *Vibrio* species assemblages and their spatio-temporal patterns in scleractinian corals is essential to understand the occurrence and proliferation of potential coral pathogens. Bleaching in *O. patagonica* has been studied extensively since it was first observed on the Israeli coast in 1993 and *V. shilonii*, which was subsequently described as a later heterotypic synonym of *V. mediterranei* [40], was identified as the causative agent [18,19]. Recently, *V. coralliilyticus* has also been related to *O. patagonica* diseases [24,35].

Cladocora caespitosa is the only endemic zooxanthellate scleractinian reef-building coral in the Mediterranean Sea and displays high sensitivity to thermal stress [31–33,15]. Indeed, mass mortality events of this species have been recorded during anomalous summer temperature increases throughout the Mediterranean Sea [11,5,15,16]. Although an important effort has been made to study the thermal tolerance of this species in aquaria and to compare it with *O. patagonica* [32,33], the role of vibrios in this coral species and their potential for causing warming-induced mortality have not been addressed.

In order to provide more information on the relationship between *Vibrio* species and coral mortality in the Mediterranean Sea, this present study analyzed the diversity of culturable *Vibrio* communities associated with the endemic *C. caespitosa* and the putative alien species *O. patagonica*, which are two coral species that can cohabit. In good agreement with previous results, the pathogens *V. mediterranei* and *V. coralliilyticus* were detected in diseased samples of *O. patagonica* from two different locations, supporting the bacterial bleaching hypothesis. Notably, these vibrios were also detected in *C. caespitosa* colonies that showed tissue necrosis, suggesting that these pathogens could also be implicated in this disease.

Materials and methods

Sample collection and estimation of disease signs

Samples were taken from two different locations in the Mediterranean Sea in 2012: Pietra Ligure (44°08′50.17″N, 08°17′04″W, Italy) in the Ligurian Sea, and Tabarca (38°09′59″N, 00°28′56″W, Spain) in the Balearic Sea (Fig. 1). Fragments from four distinct colonies of *O. patagonica* and *C. caespitosa* (i.e. a fragment from each colony) and three samples of surrounding water (at 50 cm) were randomly collected in June. In September, when tissue necrosis was observed in some coral colonies, fragments from two healthy and two unhealthy colonies were taken together with three samples of surrounding water (Fig. 1).

The samples were immediately placed in plastic bags underwater and transported to the laboratory in a cooler. According to coral health status, corals were classified by visual inspection as healthy when they were normally pigmented and unhealthy when the percentage of bleached surface area or necrotized tissue was greater than 25%.

Coral colonies were gently washed three times with 50 mL of sterile filtered seawater (SFSW) to remove transient non-associated bacteria, broken into $2 \times 2\ cm^2$ pieces, and crushed in SFSW using a pestle and mortar. Then, the skeleton was allowed to settle for 15 min and the supernatant (crushed tissue) was removed and used for *Vibrio* isolation.

Isolation of Vibrio *spp. and DNA extraction*

For plate counts of *Vibrio* spp., 1 mL of seawater and crushed coral tissue supernantant were used to prepare serial dilutions in SFSW that were then plated in triplicate on thiosulphate citrate bile sucrose (TCBS) agar (Pronadisa, Spain) and marine agar (MA) (Pronadisa). All plates were incubated at 30 °C for 48 h. Different colony morphotypes were identified on the basis of color, size and morphology. Three colonies of each morphotype were randomly selected, re-streaked onto fresh plates, and incubated for a further 48 h at 30 °C. The process was repeated three times until pure cultures were obtained. Colonies isolated from MA were tested for Gram-negative staining and fermentative glucose metabolism by the O/F test (Pronadisa, Spain) in order to select the isolates that were potentially members of the *Vibrio* genus.

DNA was extracted from each *Vibrio* isolate by boiling. In brief, a colony was suspended in 400 μL sterile Milli-Q water, heated to 99 °C in a dry block (Thermomixer Compact, Eppendorf) for 10 min and centrifuged at $13{,}000 \times g$ for 10 min (Biofuge Pico, Heraeus Instruments), and the supernatant was then used as template DNA for PCR.

MALDI-TOF MS analyses and identification by 16S rRNA gene sequencing

An initial screening of the isolated strains was carried out with MALDI-TOF MS, using whole cell biomass, as previously described by Viver et al. [44]. Reference strains (*V. mediterranei*–*V. shilonii* AK-1 CECT 7873, *V. mediterranei* CECT 621^T, *V. coralliilyticus* LMG 20984^T, *V. tubiashii* LMG 10936^T, *V. splendidus* CECT 618 and *V. breoganii* CECT 7367) were also added to the analysis. Profiles were grouped into similarity clusters. Each profile was identified with a score that matched the references divided into four ranges: highly probable species identification (>2.3), probable species identification (2.0–2.3), reliable genus identification (1.7–2.0), and unreliable identification (<1.7). The identity of the members of each cluster was further confirmed by 16S rRNA gene sequence analysis.

16S rRNA genes from one or two strains of each single similarity cluster identified by MALDI-TOF were sequenced following PCR amplification with specific bacterial primers 21F and 1492R [20]. The reaction mixtures (50 μL) contained 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris–HCl (pH 9.0), 200 mM of each deoxyribonucleotide triphosphate (Invitrogen), 1U of *Taq* I DNA polymerase (Invitrogen), each oligonucleotide primer at a concentration of 2 mM and 2 μL of template DNA. Amplification conditions included an initial denaturation step at 95 °C for 3 min followed by 30 cycles at 94 °C for 15 s, 55 °C for 30 s, 72 °C for 2 min and a final extension step at 72 °C for 7 min. Negative controls without template DNA were also included. PCR products were checked on 1% agarose gels (LE; SeaKem) in 1× Tris–acetic acid-EDTA (TAE) buffer and visualized under UV light after ethidium bromide staining.

PCR products were purified using the GeneJET^{TM} PCR purification kit (Fermentas, EU), according to the manufacturer's protocol, and sequenced using an ABI 3730xl sequencer (Applied Biosystems). The analyses of the almost complete 16S rRNA gene sequences from 20 strains were conducted using the ARB software package [23] with the reference databases LTPs123 (LTP project, [26]; http://www.arb-silva.de/projects/living-tree) and SSU Ref123 (SILVA project, [29]; http://www.arb-silva.de). Sequences were automatically aligned using the SINA software [27], followed by manual inspection of misplaced bases using the ARB sequence editor taking into account the secondary structure of the 16S rRNA gene. Tree reconstructions were performed with the almost

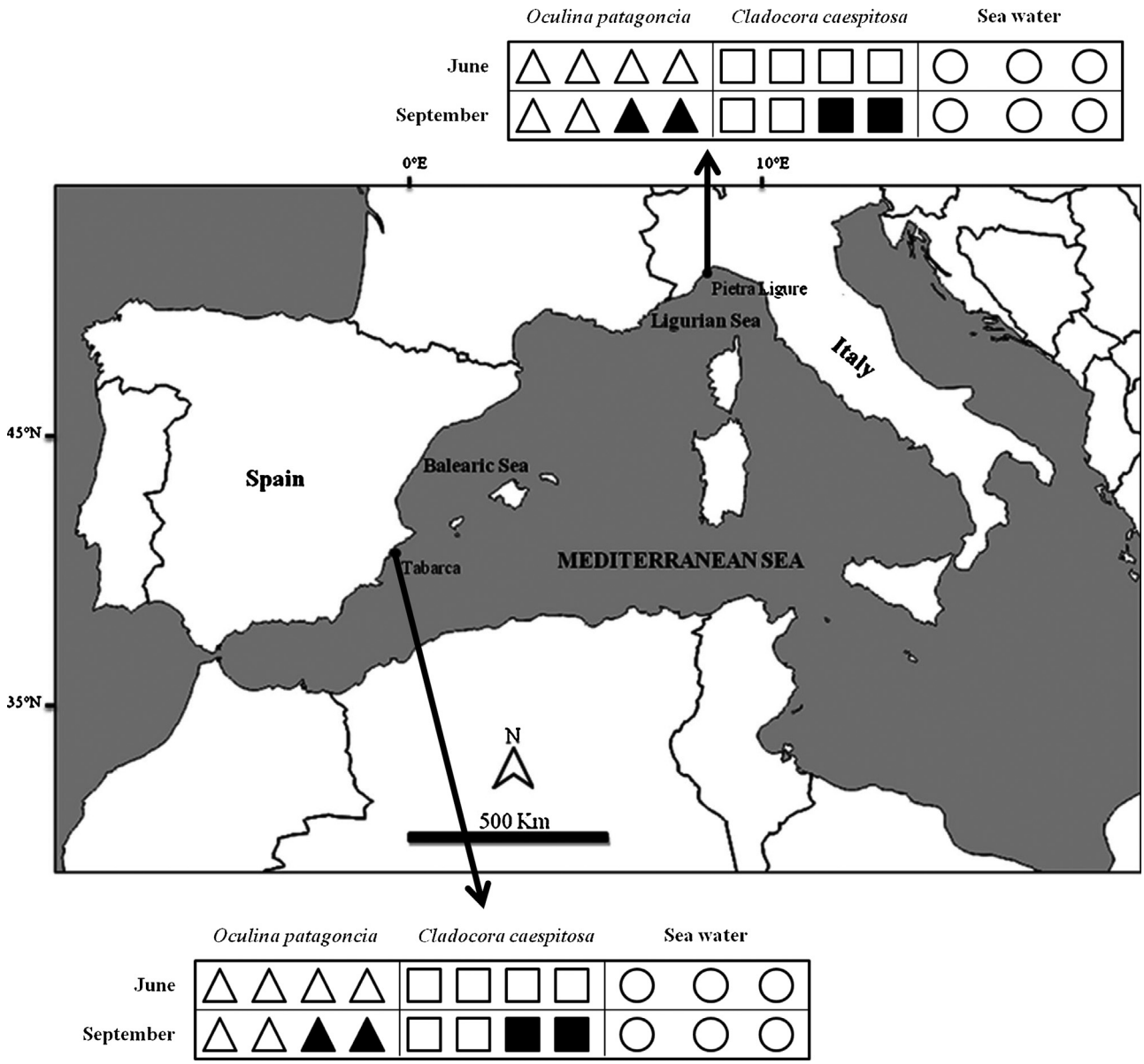

Fig. 1. Geographic areas sampled in this study and samples taken at each sampling time. Samples are indicated for water (circles), *Oculina patagonica* (triangles) and *Cladocora caespitosa* (squares), with black for unhealthy samples and white for healthy samples.

complete sequences using the neighbor-joining algorithm with Jukes–Cantor correction and the 30% maximum frequency filter to remove noise from the alignment and additionally to guarantee positional orthology. Partial sequences were added to the reconstructed reference tree using the ARB parsimony tool. The sequences have been deposited in the GenBank repository under the accession numbers KX279474–KX279521.

Random amplified polymorphic DNA (RAPD) typing

In order to assess the clonality of the strains belonging to the same phylotypes, strains were typed by RAPD using the PB1 primer (5′-GCGCTGGCTCAG-3′), as previously described [30]. The reaction mixtures (25 μL) contained 2.5 μL 10× PCR reaction buffer (Invitrogen), 2 μL 50 mM $MgCl_2$, 0.5 μL dNTPs mixture, 0.5 μL 100 μM PB1 primer and 0.5 units of Taq polymerase, with sterile Milli-Q water up to 25 μL and 250 ng DNA from each *Vibrio* strain as template. The following PCR conditions were used: one cycle of 94 °C for 5 min, followed by 10 cycles at 94 °C for 1 min, 40 °C for 1 min, 72 °C for 1 min, 22 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min, and one cycle at 72 °C for 4 min. Gel electrophoresis of PCR products was performed in a 2% agarose gel (LE; SeaKem) at 110 V for 120 min, using Lambda HindIII as a molecular weight marker (Invitrogen). Gels were stained with ethidium bromide, then visualized and photographed under UV light. Images were imported into PyElph software [27] for analysis and the bands obtained were recorded in binary code (presence = 1, absence = 0). The same reference strains as in the MALDI-TOF MS experiment were used. Dendrograms were created using the UPGMA method and strains that clustered above 90% were considered to belong to the same clonal type [14].

Data analysis

Multivariate analyses performed with a Primer 5 software package [7] were used to compare the composition and diversity of *Vibrio* spp. communities associated with different samples in order to determine which phylotypes were specific to, or shared between,

Table 1
Total bacteria and *Vibrio* spp. counts from three replicates of each sample for seawater and corals at both sampling locations. (CFU): colony-forming units.

				Total bacteria counts on Marine Agar (CFUs)	*Vibrio* spp. counts on TCBS (CFUs)	*Vibrio* percentage
Spain	Water	June		$1.56 \times 10^4 \pm 2.13 \times 10^3$	$1.33 \times 10^2 \pm 5.77 \times 10^1$	0.916
		September		$3.17 \times 10^5 \pm 4.26 \times 10^4$	$3.77 \times 10^3 \pm 2.65 \times 10^2$	1.18
	O. patagonica	June		$1.02 \times 10^5 \pm 2.01 \times 10^4$	$2.73 \times 10^3 \pm 6.34 \times 10^2$	2.67
		September	Healthy	$7.40 \times 10^5 \pm 3.78 \times 10^4$	$5.32 \times 10^4 \pm 2.80 \times 10^3$	7.18
			Unhealthy	$8.72 \times 10^5 \pm 5.17 \times 10^4$	$2.08 \times 10^5 \pm 5.74 \times 10^4$	23.85
	C. caespitosa	June		$1.92 \times 10^6 \pm 9.03 \times 10^4$	$6.69 \times 10^4 \pm 3.68 \times 10^3$	3.48
		September	Healthy	$1.78 \times 10^6 \pm 1.31 \times 10^5$	$8.78 \times 10^4 \pm 4.08 \times 10^3$	4.93
			Unhealthy	$1.62 \times 10^6 \pm 9.87 \times 10^4$	$5.20 \times 10^5 \pm 6.35 \times 10^4$	32.09
Italy	Water	June		$8.06 \times 10^3 \pm 5.28 \times 10^2$	$8.07 \times 10^1 \pm 5.81 \times 10$	1.00
		September		$7.63 \times 10^4 \pm 3.77 \times 10^3$	$8.43 \times 10^2 \pm 3.48 \times 10^1$	1.10
	O. patagonica	June		$2.21 \times 10^4 \pm 1.29 \times 10^3$	$8.43 \times 10^2 \pm 3.48 \times 10^1$	3.81
		September	Healthy	$8.11 \times 10^5 \pm 6.54 \times 10^4$	$6.32 \times 10^4 \pm 1.25 \times 10^4$	7.79
			Unhealthy	$5.91 \times 10^5 \pm 2.62 \times 10^4$	$1.14 \times 10^5 \pm 2.09 \times 10^4$	19.28
	C. caespitosa	June		$3.78 \times 10^5 \pm 1.62 \times 10^4$	$1.38 \times 10^4 \pm 2.14 \times 10^3$	3.65
		September	Healthy	$9.39 \times 10^5 \pm 6.05 \times 10^4$	$9.80 \times 10^4 \pm 1.70 \times 10^4$	8.74
			Unhealthy	$9.11 \times 10^5 \pm 7.55 \times 10^4$	$2.36 \times 10^5 \pm 9.93 \times 10^4$	28.10

localities or coral samples. A distance matrix was constructed using Bray–Curtis similarity, and non-metric multidimensional scaling (NMDS) was used to explore the groupings of the samples. Analyses of similarity (ANOSIM) were carried out to determine whether sampling location (Italy/Spain), coral species (*C. caespitosa*/*O. patagonica*), sampling time (June/September) or coral status (healthy/unhealthy) had an effect on the *Vibrio* spp. communities. Similarity percentage (SIMPER) was used to identify phylotypes that could be potentially responsible for these differences.

Results

Overall Vibrio *community analyses*

A total of 16 colonies of *C. caespitosa* and *O. patagonica* (12 healthy and 4 showing signs of necrosis, see Fig. 1) and 6 water samples collected from Pietra Ligure and Tabarca were analyzed. The presence and relative abundance of culturable vibrios were assessed in all samples by comparing the concentrations of *Vibrio* species and total culturable marine bacteria. For both locations, *Vibrio* spp. counts were always higher in corals than in seawater and their numbers increased from June to September (Table 1). In both cases, culturable *Vibrio* spp. constituted a small part of the total culturable bacterial community (i.e. colonies recovered on marine agar): approximately 1% in seawater and 4% in healthy corals. Among the samples collected in September at both localities, unhealthy corals from the two coral species yielded bacterial counts more than five times higher than healthy corals (Table 1) and the proportion of *Vibrio* spp. reached 20% and 30% of the culturable bacteria in *O. patagonica* and *C. caespitosa*, respectively. In addition, the *Vibrio* assemblage was more diverse (according to H' values) in corals than in water samples (t-test, $p < 0.001$) and slightly more diverse in *C. caespitosa* than in *O. patagonica* (Table 2).

On the basis of morphological traits, a total of 167 *Vibrio* isolates were obtained and grouped by MALDI-TOF MS into 15 distinct profile clusters (Fig. S1). Each cluster was identified according to the 16S rRNA gene affiliation (Table 3 and Fig. S2). Since each cluster was formed by organisms affiliating with the same lineage, each one was considered to be a phylotype, although it also corresponded to a single species. *Vibrio* compositions were significantly different between geographic sites and between corals and water, as shown by nMDS (Fig. 2) and confirmed by ANOSIM ($R = 0.346$, $p = 0.003$). Furthermore, a 2-way crossed ANOSIM test (locality × coral host) indicated that there were no differences in *Vibrio* species compositions between coral species at a given location ($R = 0.892$, $p > 0.05$), and that *Vibrio* assemblages from unhealthy corals tended to converge independently from the geographical origin (see Fig. 2).

Only six different phylotypes were retrieved from seawater samples. Among them, *V. chagasii* and *V. owensii* were the most frequently isolated vibrios in samples from Italy and Spain, respectively. Corals harbored a higher *Vibrio* diversity, since a total of 14 distinct phylotypes were detected. In this case, the most frequently retrieved were members of the *V. splendidus* super-clade and *V. sinaloensis* in *O. patagonica,* and *V. breoganii*/*comitans* in *C. caespitosa* (see Table 3).

Coral culturable Vibrio *communities and geographic location*

According to the SIMPER results (Table 4a), the main phylotypes responsible for the geographic differences observed in corals were the *V. splendidus* super-clade and *V. owensii*, which were most frequently retrieved from samples collected in the Spanish samples, and *V. sinaloensis*, *V. rotiferianus* and *V. chagasii* that were mainly present in samples from Italy.

Furthermore, as mentioned above, although ANOSIM analysis did not show significant differences between the *Vibrio* communities harbored by the two coral hosts, due to the intraspecies variability, these communities were not identical and there were some *Vibrio* phylotypes that showed strong host-coral specificity. This was the case for *V. breoganii*/*comitans* and *V. chagasii* that were mostly associated with *C. caespitosa* and rarely retrieved from *O. patagonica* samples or seawater. Conversely, *Photobacterium rosenbergii* was only isolated from two samples of *O. patagonica.* The rest of the phylotypes were present in both coral hosts.

Vibrio *assemblages and coral health status*

SIMPER analysis (Table 4b) also revealed that the health status at both sampling locations had a strong effect on the detected *Vibrio* phylotypes. Thus, while healthy samples of *O. patagonica* and *C. caespitosa* were dominated by the *V. splendidus* super-clade and *V. breoganii*/*comitans*, respectively, unhealthy corals were dominated by phylotypes closely related to the coral pathogens *V. mediterranei* and *V. coralliilyticus*. In order to obtain further information concerning the diversity of these four phylotypes that were responsible for the differences between healthy and unhealthy corals, the corresponding 61 strains (Table 3) were compared by RAPD. As shown in Fig. S3, each phylotype showed reproducible patterns that contained from seven to twelve bands.

RAPD analysis showed that the *V. splendidus* super-clade and *V. breoganii*/*comitans*, which were mainly related to healthy samples, harbored a considerable heterogeneity at the strain level, with

Table 2
Vibrio diversity in coral samples and seawater used in this study. Diversity indices were calculated from phylotypes retrieved from each sample and identified by MALDI-TOF.

Habitat	Locality	Time	Coral status	T	*Vibrio* diversity (H′)
Oculina patagonica	Italy	June	Healthy	24.2	1.670
		September	Healthy	26.8	1.089
			Unhealthy		1.479
	Spain	June	Healthy	24.2	1.540
		September	Healthy	27.3	1.435
			Unhealthy		1.951
Cladocora caespitosa	Italy	June	Healthy	24.2	1.990
		September	Healthy	26.8	1.749
			Unhealthy		2.069
	Spain	June	Healthy	24.2	1.748
		September	Healthy	27.3	1.667
			Unhealthy		1.758
Water	Italy	June		24.2	0.691
		September		26.8	1.099
	Spain	June		23.9	0.691
		September		27.3	1.043

Table 3
Vibrio phylotypes, identified by MALDI-TOF and named based on 16S rRNA results, retrieved from corals *Cladocora caespitosa* and *Oculina patagonica* (H: Healthy; U: Unhealthy), and seawater samples collected in Italy and Spain.

Phylotype number	Identification name	Locality	Water	*Cladocora caespitosa*		*Oculina patagonica*		Total
				H	U	H	U	
1	*Vibrio* sp. 1	Spain	–	1	–	–	1	2
		Italy	1	1	–	2	–	4
2	*Photobacterium rosenbergii*	Italy	–	–	–	2	–	2
3	*Vibrio*	Spain	–	6	–	1	–	7
	breoganii/comitans	Italy	–	4	–	–	–	4
4	*Vibrio maritimus*	Spain	–	1	1	1	–	3
		Italy	–	1	2	1	–	4
5	*Vibrio mediterranei*	Spain	–	–	2	–	1	3
		Italy	–	–	1	–	2	3
6	*Vibrio ponticus*	Italy	–	–	1	–	–	1
7	*Vibrio coralliilyticus*	Spain	–	1	3	–	2	6
		Italy	–	3	5	–	6	14
8	*Vibrio sinaloensis*	Spain	–	1	–	1		2
		Italy	–	5	1	3	1	10
9	*Vibrio fortis*	Spain	–	2	–	2	1–	5
		Italy	1	–	1	1	1	4
10	*Vibrio chagasii*	Italy	5	2	2	1	–	10
11	*Vibrio caribbeanicus*	Spain	–	2	–	3	–	5
		Italy	1	–	2	–	–	3
12	*Vibrio owensii*	Spain	7	4	3	4	1	19
		Italy	1	2	–	1	–	4
13	*Vibrio splendidus*	Spain	1	3	1	8	1	14
	super-clade	Italy	3	1	1	4	1	10
14	*Vibrio rotiferianus*	Spain	–	–	1			1
		Italy		3	3	4	1	11
15	*Vibrio harveyi*	Spain	–	–	–	2	2	4
		Italy	–	3	–	2	1	6

19 and 7 distinct clonal types identified at 90% similarity, respectively. It was remarkable that identical clones of the *V. splendidus* super-clade (in *O. patagonica*) and *V. breoganii*/*comitans* (in *C. caespitosa*) were detected in samples from the two different sampling locations (Fig. 3A and 3B). In addition, identical clonal types were detected for the coral pathogens *V. mediterranei* and *V. coralliilyticus* in both coral species. However, the *V. mediterranei* isolates were more diverse in the Italian samples, while only one clonal type was retrieved from Spain, which was not recovered from Italy. This clonal type was close to strain AK1, previously related to *O. patagonica* bleaching in Israel [21] (Fig. 3C). *V. coralliilyticus* strains were mainly retrieved from samples with signs of necrosis taken in Italy, but two clonal types were detected simultaneously at both sampling locations. Noticeably, some strains of this well-known pathogen were retrieved from healthy *C. caespitosa* samples in Italy and they all belonged to the same clonal type only found in healthy colonies, since they were different to the clonal types retrieved from unhealthy samples (Fig. 3D).

Discussion

In this study, the *Vibrio* community associated with the Mediterranean endemic coral *C. caespitosa* was analyzed for the first time and compared with that of the putatively invasive coral *O. patagonica*. Although the identification of vibrios at the species level poses considerable challenges due to the limitations of using 16S rRNA gene phylogeny in this group [12], the phylotype identification was supported not only by 16S rRNA gene sequencing but by RAPD and MALDI-TOF MS analyses, which had been successfully used by Dieckmann et al. [9] and Erler et al. [10] to identify *Vibrio* species.

The identification of the core microbiome (i.e. the members shared among microbial consortia from similar habitats) is of paramount relevance for understanding the stable and consistent components across complex microbial assemblages. The results of this study supported the view that corals harbored different *Vibrio* strains as members of their core microbial communities [1].

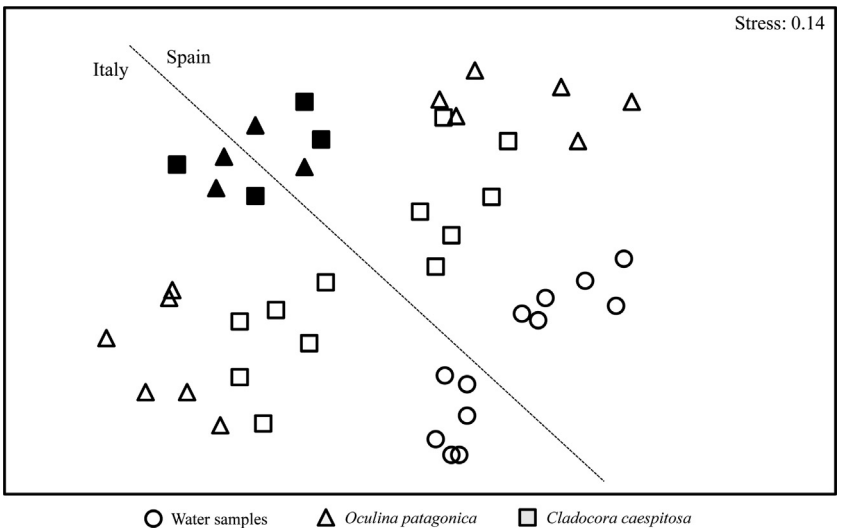

Fig. 2. Non-metric multidimensional scaling plot of the first two dimensions based on Bray–Curtis dissimilarities. Samples are indicated for water (circles), *Oculina patagonica* (triangles) and *Cladocora caespitosa* (squares), with black for unhealthy samples and white for healthy samples.

Table 4
Percentage contribution of each phylotype to *Vibrio* community structure (based on SIMPER analysis), indicating the average contribution to the similarity (S) within each grouping factor and the dissimilarity (D) between them: (a) localities and (b) among locality coral heath status in each (H: healthy and U: Unhealthy corals) . Phylotypes with a percentage contribution higher than 20 are in bold.

a) Locality

Phylotype	Italy	Spain	Italy/Spain
	S = 49.12	S = 38.03	D = 74.56
Vibrio splendidus super-clade	–	**47.30**	16.55
Vibrio owensii	5.47	15.49	5.52
Vibrio sp. 2	–	11.40	5.03
Vibrio sinaloensis/gazogenes	**22.42**	–	14.96
Vibrio rotiferianus	7.34	–	10.09
Vibrio chagasii	6.27	–	9.11
Vibrio coralliilyticus	5.42	2.58	7.59

b) Locality × coral health status

Phylotype	Italy			Spain		
	H	U	H/U	H	U	H/U
	S = 38.66	S = 57.14	S = 69.15	S = 38.07	S = 60.09	S = 67.80
Vibrio sinaloensis/gazogenes	7.35	5.13	4.16	–	–	–
Vibrio comitans/breoganii	**19.74**	–	6.03	9.90	–	4.63
Vibrio harveyi-like	7.61	12.50	8.30	–	11.11	10.54
Vibrio splendidus super-clade	**37.35**	–	8.16	**43.56**	–	14.50
Vibrio coralliilyticus	–	**46.21**	26.91	–	**33.32**	21.87
Vibrio mediterranei	–	**20.36**	10.14	–	**30.45**	9.92

Thus, identifying *Vibrio* species that constitute such core communities is the first step in defining a 'healthy' microbial community and predicting community responses to disease. In the two coral species analyzed, two main dominant phylotypes were identified that could be part of their 'core' microbiota: the *V. splendidus* super-clade in *O. patagonica* and *V. breoganii/comitans* in *C. caespitosa*. The *V. splendidus* super-clade related species had been detected previously in *O. patagonica* as one of the most abundant taxa present all year round in this coral on Israeli [17] and Spanish coasts [36]. Furthermore, in the present study, identical clones of this phylotype have been retrieved from *O. patagonica* colonies hundreds of kilometers apart. Thus, this bacterial taxon seems to be closely associated with the coral *O. patagonica* throughout the Mediterranean Sea, supporting the idea that it could be part of the coral's 'core microbiome' as a symbiont and could have a relevant role in the coral holobiont, such as conferring protection against further infection through antimicrobial activity [38]. Conversely, the *V. breoganii/comitans* phylotype seemed to be closely associated with healthy specimens of the endemic coral and, therefore, it likely belonged to the 'core' *C. caespitosa* microbiota.

On the other hand, unhealthy samples of *O. patagonica* and *C. caespitosa* harbored similar *Vibrio* communities and shared clonal types of the two coral pathogens *V. coralliilyticus* and *V. mediterranei*, even at different geographic locations in the Mediterranean Sea (see Fig. 3). This is the first time that the presence of both pathogens has been reported in unhealthy samples of *C. caespitosa*, including a *V. mediterranei* clonal type shared with *O. patagonica* closely related to strain AK1 (=*V. shilonii*), suggesting that these pathogens could also be related possibly to tissue necrosis of this species. This hypothesis was also supported using PCR for the detection of *V. coralliilyticus* in all unhealthy samples of *C. caespitosa* collected from Italy (data not shown), with primers developed for diagnostic detection of the zinc-metalloprotease gene [45] involved in the virulence of this coral pathogen. These results together with the repeated report of the presence of these pathogens in unhealthy samples of different species of Mediterranean corals [3,4,35,36]

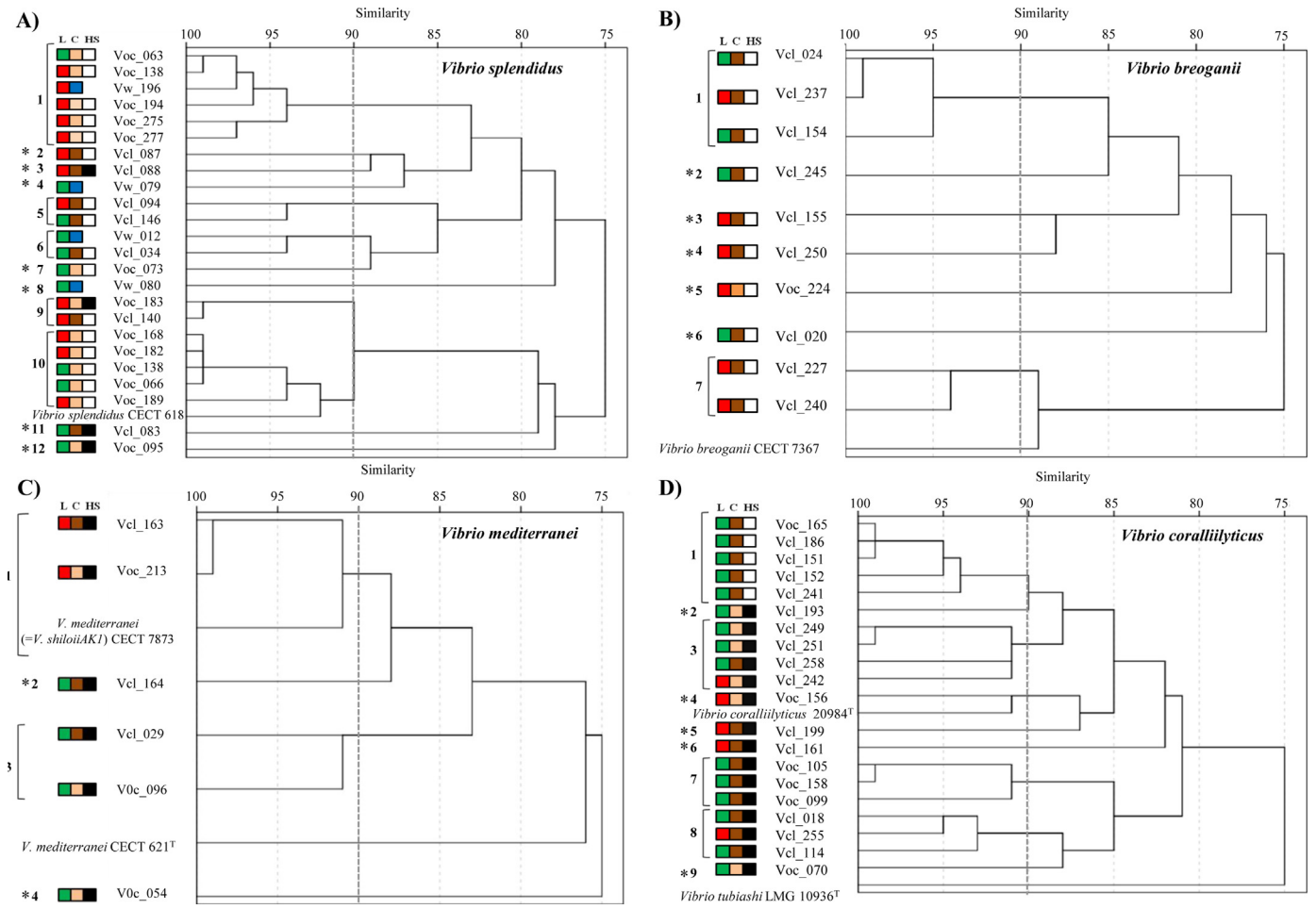

Fig. 3. UPGMA clustering of *Vibrio splendidus* (A), *V. breoganii* (B), *V. mediterranei* (C) and *V. coralliilyticus* (D) related strains, with type strains *V. splendidus* CECT 618, *V. breoganii* CECT 7367, *V. mediterranei–V. shilonii* AK-1 CECT 7873, *V. mediterranei* CECT 621T, *V. coralliilyticus* LMG20984T and *V. tubiahii* LMG10936T as references, showing genetic relatedness of the strains using random amplified polymorphic DNA (RAPD) PCR. * single clones. Location: Spain in red and Italy in green; Coral species: *Cladoccora caespitosa* in dark brown and *Oculina patagoncia* in light brown; and health status: healthy in white and unhealthy in black.

suggested that they could be generalist pathogens in the Mediterranean Sea.

In contrast to *V. mediterranei* that was only retrieved from unhealthy samples, *V. coralliilyticus* was also isolated from apparently healthy *C. caespitosa* samples (taken in June from Genova) and its presence was also confirmed by zinc-metalloprotease gene-specific PCR. However, the clonal type isolated from the healthy specimens was different from that retrieved from unhealthy organisms, and this may suggest that not all strains of this species have the same level of virulence. In fact, previous studies with human pathogens have demonstrated that genome variation is important in the ability of pathogens to interact with the host, so the invasive-disease potential for a strain can be affected by its clonal type, such as that observed in *Streptococcus pneumoniae* [25] or *Staphylococcus aureus* [37]. Furthermore, coral diseases not only depend on the presence of *Vibrio* coral pathogens and their virulence level, but are also the result of complex interactions between the expression of different bacterial virulence factors and an increase of seawater temperature or other environmental stresses, as well as the physiological and immune status of the coral host.

Concluding remarks

Overall, the most remarkable result from this study was the fact that healthy corals showed *Vibrio* communities that were diverse and distinct depending on the geographical location of the coral origin. However, the diversity decreased in unhealthy corals, and the community composition became more homogeneous between corals independently of the origin of the specimens. Thus, different Mediterranean coral species (*P. clavata*, *O. patagonica*, and now *C. caespitosa*) seemed to share the same pathogens, namely *V. coralliilyticus* and *V. mediterranei*. However, in the light of these results, the presence of *V. coralliilyticus* was not always related to the development of disease signs in *C. caespitosa*, which could indicate that the bacterium clonal variation could have an influence on its virulence. Even though the origin of *O. patagonica* is unclear [22], its demonstrated spread through the Mediterranean Sea could enhance secondary horizontal *Vibrio* transmission by physical contact between neighboring corals of different species. Therefore, the factors affecting the spread of any given coral species could have previously unanticipated effects on the health of the coral community as a whole.

Acknowledgements

The authors gratefully thank the staff of the Department of Marine Sciences and Applied Biology and the Marine Research Centre of Santa Pola (CIMAR). We also greatly appreciate the friendly cooperation of the Secretary-General for Fisheries of the Spanish Ministry of Agriculture, Food and Environment, and the Marine

Reserve of Tabarca wardens (particularly Felio Lozano). Andres Izquierdo and Marco Palma assisted with sample collection, Maria Jesus Pujalte assisted with *Vibrio* identification, Nuria Sarriá helped carry out RAPD, and Guido Jones reviewed the English version. We are also grateful to the University of Alicante for awarding a predoctorate grant to Esther Rubio-Portillo. This work was funded by the European Union's Horizon 2020 framework program (LEIT-BIO-2015-685474, Metafluidics, to JA) and the grant CLG2015_66686-C3-3 (to JA) of the Spanish Ministry of Economy and Competitiveness, which was also co-financed with FEDER support from the European Union.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.syapm.2017.11.007.

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