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Full Length Research Paper

Molecular typing of *Vibrio alginolyticus* strains isolated from Tunisian marine biotopes by two PCR-based methods (ERIC and REP)

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The aim of the present study was to analyze the intraspecific genetic variability within *Vibrio alginolyticus* isolated strains from different Tunisian aquatic biotopes by using two PCR–based techniques. Seventy eight strains including nine *Vibrio* reference strains and sixty nine *V. alginolyticus* isolated strains from seawater, sediment, fish (diseased Gilthead seabream, *Sparus aurata* and Sea bass, *Dicentrachus labrax*), and from mollusks (*Crassostrea gigas*, *Mytilis edulis*) were analyzed by repetitive extragenic palindromic PCR (REP-PCR) and enterobacterial repetitive intergenic consensus sequence PCR (ERIC-PCR). In this study, all strains were typable y in both techniques tested and 62 genotypes were generated by ERIC-PCR and 58 genotypes by REP-PCR. In conclusion, these two methods can be used as a rapid and easy protocol to access the genetic relatedness between environmental *V. alginolyticus* strains.

Key words: *Vibrio alginolyticus*, genetic diversity, repetitive extragenic palindromic PCR (REP-PCR), enterobacterial repetitive intergenic consensus sequence PCR (ERIC-PCR).

INTRODUCTION

The members of genus *Vibrio* are autochthonous to aquatic environments including estuarine, coastal waters and sediments worldwide, and some species are well-known pathogens of marine organisms including fish, crustaceans, corals and mollusks (Gomez-Gil et al., 2005, 2005; Hidalgo et al., 2008; Balcazar et al., 2010). Halophilic *Vibrio* causes infection disease in humans by consuming seafood. Outbreaks of food toxic infection have been recorded in many geographical areas such as: Japan, countries of Southeast Asia, the United States, England, Australia, India and other countries (Beleneva et al., 2004). Of the genus *Vibrio*, the most thoroughly studied species is *V. cholerae*, the etiological agent of cholera. However, in recent years, attention has focused on the halophilic non-cholera vibrios such as *V. vulnificus, V. parahaemolyticus, V. mimicus,* and *V. alginolyticus* which are frequently associated with human gastroenteritis cases mainly caused by the ingestion of contaminated water and undercooked seafood (Carburlotto et al., 2010). *Vibrio alginolyticus* is also considered as marine fish and shellfish pathogen (Gomez-Leon et al., 2005). This bacterium causes many epizootic outbreaks in gilthead sea bream (*Sparus aurata* L) and sea bass (*Dicentrarchus labrax*) which are marine fish with high economic value in the Mediterranean aquaculture (Bakhrouf et al., 1995; Zorilla et al., 2003a). As consequence, *V. alginolyticus* is responsible for mass stock mortality in many fish farms throughout the Mediterranean area (Ben Kahla-Nakbi et al., 2006; Snoussi et al., 2008). Vibrios associated with infection in bivalve mollusks belong to species *V. tapetis, V. crassostreae, V. alginolyticus* and *V. pectenicida*

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represent the most important species which cause infection in bivalve mollusks (Paillard et al., 1994;Lambert et al., 1998). *Vibrio alginolyticus* was also isolated from oysters *Crassostrea gigas* and mussels *Mytilis edulis* (Snoussi et al., 2010) and has caused mass mortalities in juvenile of *Ruditapes decussatus* (Gomez-Gil et al., 2005a, 2005b).

Knowing that biochemical methods currently used to identify *Vibrio* species can be time-consuming, labour-intensive and hard to implement because of difficulties in distinguishing between closely related species given the wide variety of biochemical profiles obtained, attention has focused on the application of molecular-based techniques. Protocols for bacterial typing using polymerase chain reaction (PCR) techniques are becoming increasingly valuable (Balcazar et al., 2010).

Several molecular typing techniques have been used and are specifically useful to study the microbial epidemiology and ecology of *Vibrio* species (Thompson et al., 2004). The molecular typing methods are necessary owing to the fact that they allow the typing of organisms on the basis of their nucleic acids; these techniques give information on the genetic relatedness of strains, the source of infection and detection of particularly virulent strains, as well as the study of the geographical and host distribution of possible variants of a specific pathogen (Olive and Bean, 1999). RAPD-, ERIC- and REP-PCR have been used for typing of *V. parahaemolyticus* (Maluping et al., 2005). These three methods were used also for the typing of *V. tapetis* (Rodriguez et al., 2006). REP-PCR has been recently used for typing *V. alginolyticus* and *V. vulnificus* isolated from diseased captive-bred seahorses and has proven to be a powerful tool for microbial ecology studies, environmental microbiology, molecular diagnostics, medical microbiology and epidemiological analyses (Balcazar et al., 2010).

The aim of this study was to use two PCR– based techniques (ERIC- and REP-PCR) for analysis of genetic variability among *V. alginolyticus* strains, taking into account their abundance in Tunisian coastal areas. These strains were recovered from fish *S. aurata* and *D. labrax*, from mollusks, *M. edulis* and *C. gigas,* and from seawater and sediment.

MATERIALS AND METHODS

Sampling sites

The strains were isolated from four marine biotopes including two fish farms where *S. aurata* and *D. labrax* are reared (Khenis and Hergla). From the conchylicole station of Menzel Jmil (*M. edulis* and *C. gigas*) and from Oued Soltane which is in connection with Mediterranean seawater during the cold seasons (Figure 1).

Seawater (500 ml) was filtered through 0.45 µm pore size filter membranes. These membranes were incubated in alkaline peptone water (1% NaCl, pH 8.6) for 24 h. The enrichments were then streaked onto Thiosulfate-citrate-bile salt-sucrose agar (TCBS agar, Bio Rad) and incubated for 18-24 and 30°C. Twenty five grams of sediment were diluted in Stomacher packet each containing 225 ml of alkaline peptone water supplemented with 1% NaCl. The homogenate was incubated at 30°C for 18 to 24 h.

Mussels and oysters were washed, scrubbed free of dirt, and then shucked with a sterile knife (Cavallo and Stabili, 2002). Twenty five grams of mussels and oysters were extracted aseptically and diluted in Stomacher packet each containing 225ml of alkaline peptone water supplemented with 1% NaCl. The homogenate was incubated at 30°C for 18 to 24 h.

Juveniles of *S. aurata* (weight: 7 g and length: 8 cm) and older fish (weight: 220 g and length: 20 cm) were obtained from a Tunisian farm installed along the Mediterranean Sea coasts (Monastir, Centre of Tunisia) during the summer 2006. The *D. labrax* specimens have been obtained from a second farm installed along the seacoasts of Hergla (Sousse, Tunisia) during the summer 2009. The clinical signs of vibriosis touching juveniles in the first hatchery included darkened body colour, white nodular skin lesion and sudden death. In the infected older fish (*S. aurata* and *D. labrax*), the visceral organs were haemorrhagic, the intestine was usually spotty and there are haemorrhages in the skeletal muscle. The ulcer was very deep and necrotic. Focal haemorrhages were also seen on the heart´s surface and the gills are usually pale. Bacteria were isolated from white nodular skin lesions associated with juveniles, water rearing basin and from the internal organs of chronically diseased older fish (Gills, blood, liver and kidney).

All samples were cultured on alkaline peptone water (1% NaCl, pH 8.6) and incubated at 30°C for 18 to 24 h. A loopful of the enrichment culture was streaked onto Thiosulphate-citrate-bile salt-sucrose agar (TCBS, Difco, Spain). After 18 to 24 h of incubation at 30°C, yellow colonies were randomly selected then subcultured on Tryptic soy agar (TSA, Difco, Spain) supplemented with 1% NaCl. Confirmation of the purity of cultures was obtained for each strain by re-streaking on tryptic soy agar 1% NaCl. The isolated bacteria were frozen at -80°C with 20% (v/v) glycerol for further analysis.

Bacterial strains

Seventy eight *Vibrio* strains were analyzed in this study including sixty nine strains of *V. alginolyticus,* six reference strains including five strains of *V. alginolyticus* (CCM 2575, CCM 2576, CCM 2578^T, ATCC 33787, ATCC 17749^T) and one *V. parahaemolyticus* strain (ATCC 43969). The two *V. parahaemolyticus* strains I_{12} and I_{14} were kindly provided by Prof. Stefania Zanetti from the Department of Biomedical Sciences (University of Sassary, Sardegna, Italy) and the *V. harveyi* (CAIM 86) strain was kindly provided by Professor Bruno Gomez Gil (CIAD, A.C., Mazatlán Unit for Aquaculture, AP. 711 Mazatlán, Sinaloa 82000, Mexico).

The strains were identified by the following biochemical tests were performed according to conventional protocols: cell morphology and motility, Gram character (KOH method: Fluharty and Packard, 1967), oxidase, growth on TCBS, susceptibility to the vibriostatic agent 0/129 (10 and 150 µg/disc), production of arginine dihydrolase, lysine and ornithine de carboxylase, glucose fermentation, indole, hydrolysis of gelatin, starch, esculin and Tween 80, reduction of nitrate to nitrite, production of gas from glucose, methyl red, growth at different temperatures (4, 37, 44°C) and at different salinities (0, 6, 8 and 10%). These tests were the main assays employed to identify the organisms belonging to *Vibrio* genus (Thompson et al., 2004).

Isolation of bacterial DNA

Isolation of DNA was performed using InstaGene Matrix (Bio-Rad, Madrid, Spain). Strains were routinely grown on trypticase soy agar (Oxoid Ltd, Madrid, Spain) plates with 1% NaCl at 37°C for 24-48 h, after which colonies were scraped off and suspended in 1 ml of

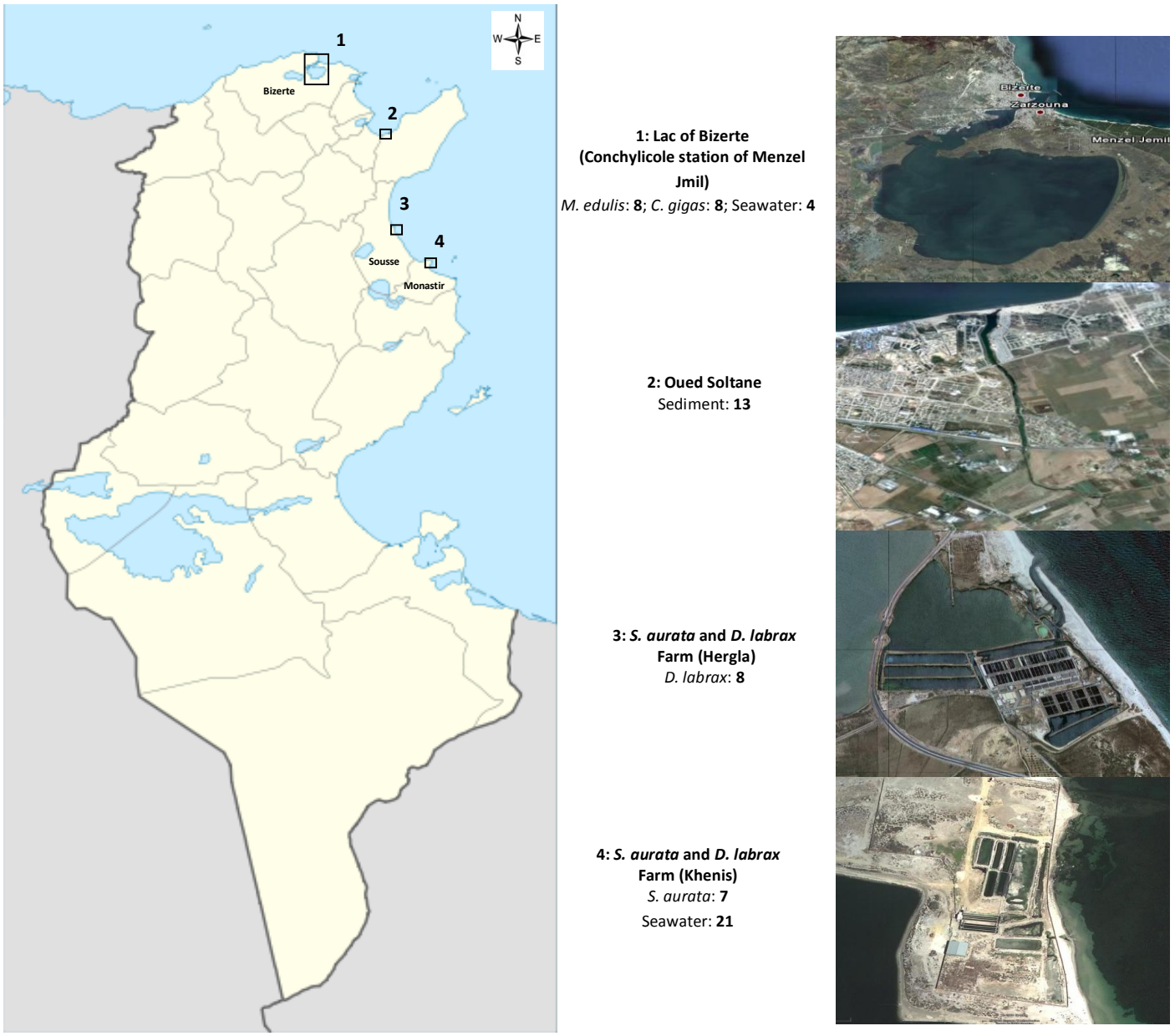

Figure 1. Map of Tunisia showing the different sites of study and the distribution of the *V. alginolyticus* strains tested in this work.

autoclaved water and centrifuged at 13 000 g for 1 min. The supernatants were removed and the remaining pellets were resuspended in 200 µl of InstaGene Matrix and incubated at 56°C for 30 min. They were then vortexed at high speed for 10 sec and boiled in a water bath for 8 min. The lysates were vortexed again at high speed and centrifuged at 13 000 g for 3 min. The InstaGene DNA preparations were stored at -20°C until used for PCR amplifications.

Molecular identification of *V. alginolyticus* strains

Confirmation of *V. alginolyticus* strains at the species level was obtained by polymerase chain reaction (PCR) using forward and reverse primers for the species-specific collagenase gene, as described previously by Di-Pinto et al. (2006).

REP-PCR typing

The REP-PCR amplifications were performed using Ready–to-Go PCR analysis beads (Amersham Biosciences). A Pair of 18-mer primers (Sigma, St Louis, MO, USA) was utilized: REP 1D (5'-NNN RCG YCG NCA TCM GGC-3') and REP 2D (5'-RCG YCT TAT CMG GCC TAC-3'), where M is A or C, R is A or G, Y is C or T, and N is any nucleotide (Stern et al., 1984). Each 25 µl REP-PCR reaction mixture contained 2.5U of Taq polymerase, 10 mmol l^{-1} Tris–HCl (pH 9), 50 mmol l^{-1} KCl, 1.5 mmol l^{-1} $MgCl_2$, 200 µmol^{-1} each deoxynucleoside triphosphate, 20 ng µl^{-1} of the respective primer, and 1 µl of DNA solution (100 µg/µl). Amplifications were performed in a T-Gradient termocycler programmed as followed: an initial denaturation step at 95°C for 7 min followed by 35 cycles of denaturation (92°C for 45 s), annealing (40°C for 1 min), and extension (72°C for 8 min), with a final extension step at 72°C for

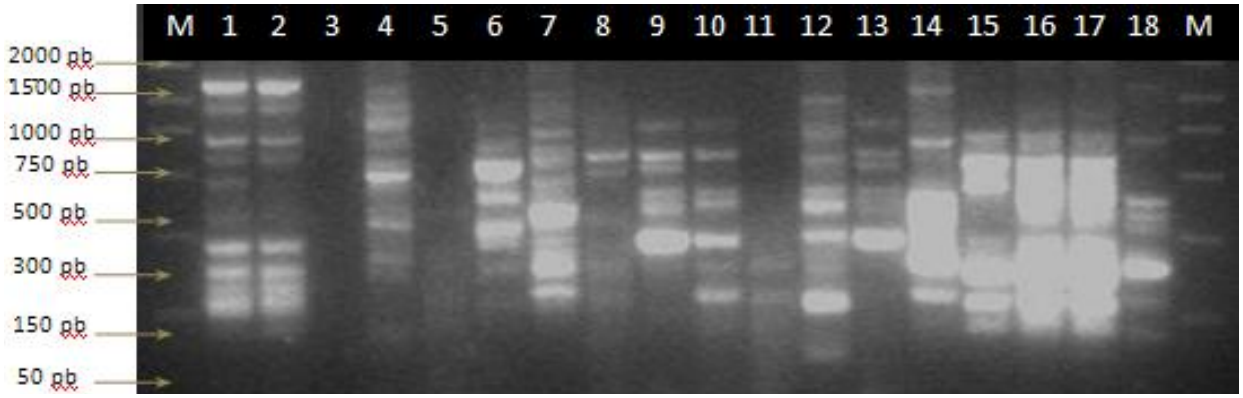

Figure 2. Strains M 50-2000 bp DNA molecular size marker; Lanes: 1: CCM2575, 2: CCM2578, 3: CCM2576, 4: CAIM86, 5:K3 (seawater), 6: H1 (sediment), 7: H3 (sediment), 8: A28 (Conchylicole station), 9: A16 (Conchylicole station), 10: S32 (aquaculture farm), 11: S 55 (aquaculture farm), 12: 126 (aquaculture farm), 13: H11 (sediment), 14: 225 (seawater), 15: P'7 (seawater), 16: A23 (Conchylicole station), 17: K8 (seawater), 18: EM3 (seawater).

15 min.

ERIC- PCR typing

The same Ready–to-Go PCR analysis beads were used for ERIC-PCR amplifications. For ERIC-PCR the following 22-mer primers (Sigma) were utilized: ERIC 1R (5'- ATG TAA GCT CCT GGG GAT TCA C-3') and ERIC 2 (5'-AAG TAA GTG ACT GGG GTG AGC G-3'). These primers were used as previously reported by Versalovic et al. (1991). After bead re-hydratation, each 25 µl ERIC-PCR reaction mixture contained 2.5 U of *Taq* polymerase, 10 mmol l^{-1} Tris–HCl (pH 9), 50 mmol l^{-1} KCl, 1.5 mmol l^{-1} $MgCl_2$, 200 µmol^{-1} each deoxynucleoside triphosphate, 20 ng $µl^{-1}$ of the respective primer, and 1µl of DNA solution (100 µg/µl). Amplifications were performed in a T–Gradient termocycler using an initial denaturation step at 95°C for 5 min followed by 35 cycles of denaturation (92°C for 45 sec), annealing (52°C for 1 min), and extension (70°C for 10 min), with a final extension step at 72°C for 20 min.

Gel electrophoresis and data analysis

Following amplification the products were analyzed by horizontal electrophoresis through 1.5% (wt/vol) agarose gel for 1h at 100V in with TAE buffer (0.04 M Tris-acetate, mM EDTA). After electrophoresis, el gel was stained with ethidium bromide (0.5 $µlml^{-1}$ gels were visualized under UV transilluminator and photographed using Gel Doc XR apparatus (Bio-Rad, USA). A negative control, consisting of the same reaction mixture but with distilled water instead of template DNA, was included in each run. DNA molecular marker (50 to 2000 bp ladder (Sigma)) was included in each electrophoresis . To determine significant differences in the patterns, the reproducibility of results was assessed by repetition of at least three independent ERIC and REP- PCR assays. The number and the size of DNA fragments were evaluated by visual inspection and using Gel Pro Analyzer 3.0 software. The data analysis was performed by using Gel Pro Analyzer 3.0 software, and the computed similarities among strains were estimated by means of the Jaccard's Coefficient (SJ). A band position tolerance value of 0.5% was allowed to compensate for misalignment of homologous bands due to technical imperfections. Cluster analysis and dendrograms were produced on the basis of the unweighted average pair group method (UPGMA).

RESULTS AND DISCUSSION

Biochemical characterization of isolated strains

In this study, twenty strains were isolated from the conchylicole station of Menzel Jmil (8 strains from *M. edulis*, 8 strains from *C. gigas* and 4 strains from seawater), thirteen strains from Oued Soltane sediment of, eight strains from *D. labrax* fish diseased in Hergla's farm (two strains from liver, three kidney strains and three gills strains) and twenty eight strains from Khenis's farm (twenty one seawater strains from four nodular skin strains juveniles associated , three liver , gills and blood strains of diseased *D. labrax* specimens).

All these strains give yellow colonies on TCBS modified agar and were identified as Gram-negative motile fermentative rods, producing enzymes like catalase and oxidase, susceptible to vibriostatic compounds O/129 (150 µg/disk) and swarming colonies on TSA 1% NaCl at different temperature: 4, 37, and 44°C. Most strains (67/69) were Voges-Proskauer and lysine decarboxylase positive. Only six strains were ornithine decarboxylase positive. However, all strains were negative for arginine dihydrolase. All, the strains grew in peptone water prepared respectively with 3, 8 and 10% of NaCl. All strains tested amplify a 737-pb size fragment showing the characteristic profile of *V. alginolyticus.*

ERIC- and REP PCR fingerprinting

Figure 2 represents the patterns obtained with ERIC-PCR tested with all studied *V. alginolyticus* population. This technique applied to 69 isolates and 9 reference strains, showed reproducible patterns consisting of 2 to 14 bands ranging from 34 to 1933 bp. The same fingerprints were observed when ERIC-PCR was repeated at least three times, demonstrating the reproducibility of the technique. Each strain gave almost a different ERIC although a

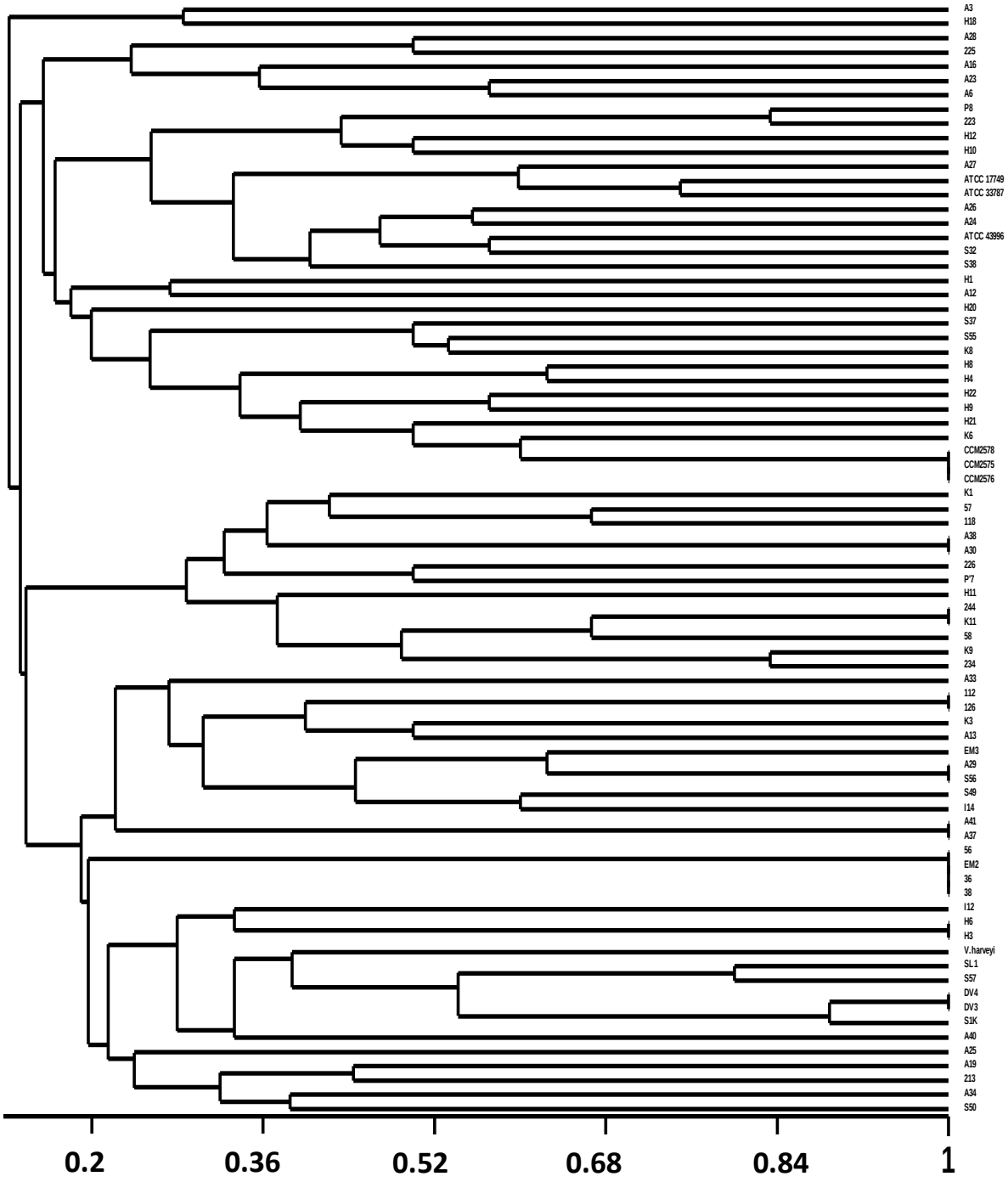

Figure 3. Dendrogram based on the unweighted pair group method of arithmetic averages and Jaccard's correlation coefficient on the basis of ERIC-PCR patterns for the 69 strains of *V. alginolyticus* isolated from seawater, sediments and from fish (*S. aurata* and *D. labrax*) and from mussels (*C. gigas* and *M. edulis*). Numbers on the horizontal axis indicate the percentage of similarity.

a common band was observed in the different strains at 500 pb. This size was also observed in previous works for *V. cholerae* isolates (Rivera et al., 1995; Rao and Surendran, 2010). Further studies would be needed to see if these bands are homologous.

The phylogenetic analysis of patterns generated by ERIC–PCR primers based on UPGMA method (Jaccard's coefficient, Figure 3), has shown that *V. alginolyticus* strains exhibit high amount of heterogeneity not only according to the origin (seawater, sediment, aquaculture farm and conchylicole station) but also within the same type of sample. In addition, two *V. alginolyticus* strains,

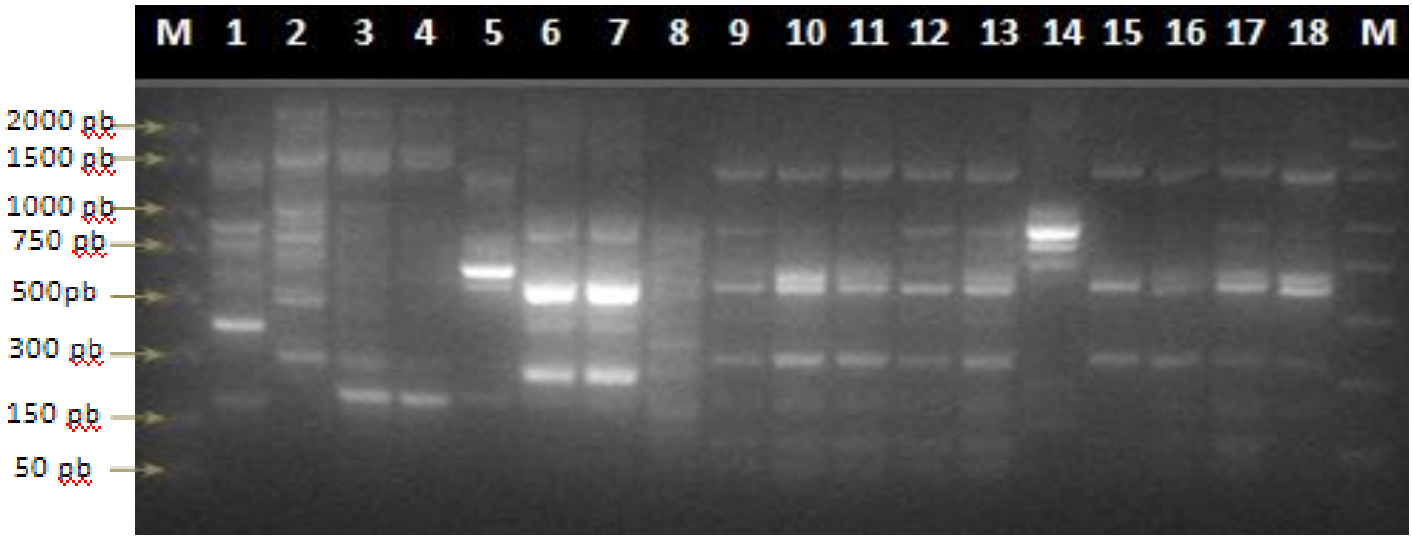

Figure 4. REP profiles *V. alginolyticus* strains. M 50-2000 bp DNA molecular size marker; Lanes 1: ATCC 43969, 2: CCM 2575, 3: CCM 2578, 4: CCM 2576, 5: *V. harveyi* CAIM 86, 6: A16 (Conchylicole station), 7: 234 (seawater), 8: H9 (sediment), 9: A6 (Conchylicole station), 10: A38 (Conchylicole station), 11: 57 (aquaculture farm), 12: 126 (aquaculture farm), 13: H11 (sediment), 14: H18 (sediment), 15: P8 (seawater), 16: K7 (seawater), 17: K9 (seawater), 18: K11 (seawater).

A29-S56, from two different sites and isolated respectively from mussels (Menzel Jmil) and from *D. labrax* (Hergla) were grouped into the same cluster and were considered as closely related strains (100% of similarity).

Figure 4 represents the patterns obtained with REP-PCR tested with some *V. alginolyticus* isolated from seawater, sediment, and aquaculture farm and conchylicole station. This technique applied to 69 isolates and 9 reference strains, showed reproducible patterns consisting of 2 to 12 bands ranging from 336 to 2688 bp. The same fingerprints were observed when REP-PCR was repeated at least three times, demonstrating the reproducibility of the technique. The analysis of patterns generated by REP–PCR primers based on UPGMA method (Jaccard's coefficient; Figure 5), has shown that *V. alginolyticus* strains exhibit high heterogeneity not only according to the origin but also within the same samples. We founded 8 clusters grouping strains closely related (100% of similarity) for the following strains (CCM 2575, CCM2576, CCM2578), (A16, A25), (K9, 57), (H11, H9), (225, 213), (EM3, EM2), (S56, S38) and (H21, H20, H18). Moreover, some strains isolated from different samples were grouped in the same cluster and were genetically identical case of K9 and k7. On the other hand, we founded that *V. alginolyticus* strains isolated from the same origin were genetically different as they were grouped in different clusters.

In this study the high intraspecific heterogeneity obtained by ERIC-PCR can be explained by the fact of the existence of different samples and different origins. Moreover, ERIC-PCR showed a higher discriminatory power than REP-PCR. In fact the former method was able to differentiate strains very closely related (100% of similarity) by REP-PCR. This result is in agreement with the findings of a previous study about molecular typing of *V. parahaemolyticus* of Maluping et al. (2005). According to these authors, the tests on repeatability showed that both RAPD and ERIC-PCR were superior than REP-PCR, their results were in accordance with several previous studies (Marshall et al., 1999; Sudheesh et al., 2002). In a recent study of Zulkifli et al. (2009), ERIC-PCR and RAPD techniques were used to determine the relationship between *V. parahaemolyticus* isolated strains on the basis of genomic fingerprinting. The result of ERIC-PCR showed a high diversity of polymorphism between *V. parahaemolyticus* isolates and concluded that this technique can be used as genome specific markers for detection of *V. parahaemolyticus* in cockles or other organisms but has to be still compared with other methods because of high level of ERIC heterogeneity detected.

Other results are in contrast such as those of Wong and Lin (2001) that preferred REP-PCR over ERIC-PCR because of the greater reproducibility of its fingerprints. In fact, the REP-PCR greatly enhances assay reproducibility and strain discrimination compared to other PCR-based platforms (Versalovic et al., 1998). This technique has proven to be highly effective for molecular typing of *V. cholerae* (Folgosa et al., 2001), *V. parahaemolyticus* (Wong et al., 2001) and *V. vulnificus* (Radu et al., 2000). In a recent study, REP-PCR was helpful for differentiating *V. alginolyticus* and *V. splendidus* strains isolated from captive bred seahorses, in epidemiological analyses, particularly in large studies and critical situations (Balcazar et al., 2010). In the same

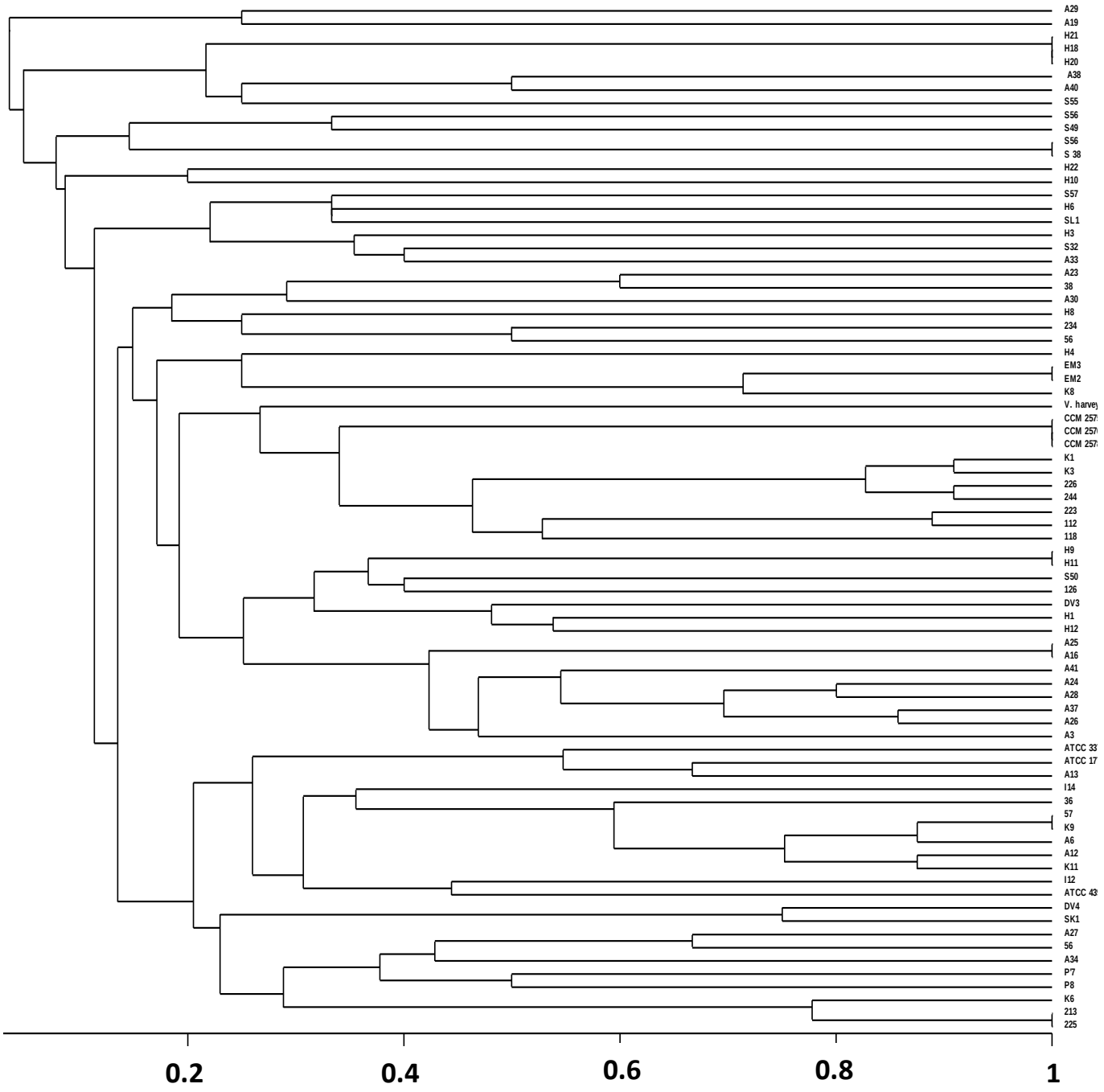

Figure 5. Dendrogram based on the unweighted pair group method of arithmetic averages and Jaccard's correlation coefficient on the basis of REP -PCR patterns for the 69 *V. alginolyticus* strains isolated from seawater, sediments and from fish (*S. aurata* and *D. labrax*) and from mussels (*C. gigas* and *M. edulis*). Numbers on the horizontal axis indicate the percentage of similarity.

research, the results obtained suggest that rep-PCR fingerprinting technique using (GTG) 5-PCR allows the differentiation of *Vibrio* strains.

Conclusions

In conclusion, results of this work have demonstrated genetic variability within the *V. alginolyticus* strains isolated from different sites of environment, seawater, and aquaculture farm and conchylicole station in Tunisia. In addition in our study, ERIC-and REP-PCR are suitable rapid typing methods for *V. alginolyticus*. The analysis of *Vibrio alginolyticus* indicates that this species is genetically heterogeneous and there was a high level of genetic diversity among strains and their recovery

sources. Both methods have a good discriminative ability and can be used as a rapid method of comparing *V. alginolyticus* strains for epidemiological investigation and for genetic diversity.

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