Determination of the Genetic Diversity of Different Bioluminescent Bacteria by Pulsed-Field Gel Electrophoresis (PFGE)

Esra Ersoy Omeroglu [1,*]

[1]Biology Department, Faculty of Science, Basic and Industrial Microbiology Section, Ege University, Bornova-Izmir, Turkey

Corresponding author: Esra Ersoy Omeroglu, Biology Department, Faculty of Science, Basic and Industrial Microbiology Section, Ege University, Bornova-Izmir, Turkey. Tel: +90-2323112811, Fax: +90-2323881036, E-mail: esraerso@gmail.com

Background: There are 4 different genera (i.e. *Vibrio*, *Aliivibrio*, *Photobacterium,* and *Shewanella*) in the new classification of bioluminescent bacteria. The mechanism of bioluminescence has yet to be fully elucidated. Therefore, the determination of physiological and genetic characteristics of bioluminescent bacteria isolated from different sources is very important. Pulsed-Field Gel Electrophoresis (PFGE) has the highest discriminatory power among the different molecular typing methods for the investigation of the clonal relationships between bacteria. For the PFGE analysis of bioluminescent bacteria, the *NotI*-HF™ is the method of choice among the restriction enzymes.
Objectives: The present study aimed to determine genetic relatedness via PFGE in 41 bioluminescent bacteria (belonging to 10 different species) isolated and identified from various marine sources.
Materials and Methods: Different bioluminescent bacteria (i.e. *Vibrio gigantis*, *V. azureus*, *V. harveyi*, *V. lentus*, *V. crassostreae*, *V. orientalis*, *Aliivibrio logei*, *A. fischeri*, *Shewanella woodyi*, and *Photobacterium kishitanii*) were analyzed by PFGE using the *NotI*-HF™ restriction enzyme. The whole DNA of the strains embedded into the agarose plugs was digested with enzyme at 37°C for 30 minutes. CHEF-Mapper PFGE system was used for electrophoresis and band profile of the strains for the *NotI*-HF™ restriction enzyme were analyzed by Bio-Profil-1D++ software (Vilber Lourmat) at 10% homology coefficient.
Results: Although all experiments were performed three times, four of forty-one bioluminescent strains (*V. gigantis* E-16, H-16 and S3W46 strains and *A. fischeri* E-4 strain) could not be typed by PFGE technique with *NotI*-HF™ enzyme. While only two strains (*V. crassostreae* H-12 and H-19 strains) were exhibiting same band pattern profiles (100% genome homology), thirty-six different PFGE band patterns were obtained. Pattern homologies changed between 66% - 92%, 73% - 83% and 49% - 100% for *V. gigantis*, *V. harveyi* and other strains, respectively.
Conclusions: The obtained results revealed that there has been a high rate of genetic diversity in bioluminescent strains isolated from Gulf of Izmir and *V. lentus* and *V. crassostreae* strains could be also bioluminescent for the first report. At the same time, PFGE analysis of bioluminescent bacteria including four different genera and ten different species were shown for the first time by this study. It is considered that data acquired by this study will contribute evolution and mechanism of bioluminescence to further works to be done.

Keywords: Pulsed-Field Gel Electrophoresis; Bioluminescent; *Aliivibrio*; *Shewanella*; *Vibrio*; *Photobacterium*

1. Background

Until recently, there were only 3 known marine bioluminescent bacterial genera (i.e. *Vibrio*, *Photobacterium,* and *Shewanella*). Nevertheless, a study performed by Urbanczyk et al. in 2007 (1) revealed that the *Vibrio fischeri* species were different from the other genera of the *Vibrionaceae* family in terms of phylogenetic and phenotypic properties. Accordingly, *V. fischeri* and *A. logei* were reclassified into a new genus called *Aliivibrio*. In addition to the new classification and according to the results of some studies, the new bioluminescent *Vibrio* and *Photobacterium* species were obtained. Up to 2007, only 15 bioluminescent species were known (i.e. *V. cholerae*, *V. fischeri*, *V. harveyi*, *A. logei*, *V. mediterranei*, *V. orientalis*, *V. splendidus* (biotype I), *V. vulnificus*, *V. salmonicida*, *Photobacterium angustum*, *P. leiognathi*, *P. phosphoreum*, *P. mandapamensis*, *Shewanella hanedai*, and *S. woodyi*) (2). Subsequent studies performed in 2007, 2009, and 2010 introduced 6 new bioluminescent species (i.e. *P. kishitanii*, *P. aquimaris*, *V. azureus*, *V. sagamiensis, V. chagasii*, and *A. sifiae*) (3-8). There was a special case for *V. chagasii*

because when it was isolated for the first time, it was indicated that this *Vibrio* species was not bioluminescent (7). A similar situation was demonstrated in a study conducted by Ersoy Omeroglu and Karaboz in 2012 (9). *Vibrio gigantis* was first isolated by Le Roux et al. in 2005 (10) and is a member of marine bioluminescent bacteria (9). In 2014, this study was cited and it was indicated that the *V. gigantis* strains were bioluminescent (11). Because of such reasons, the systematic evaluation of bioluminescent bacteria has been regularly revised in terms of their taxonomy, evolutionary relationship, and origin.

The distribution of marine bioluminescent bacteria between all bacteria has yet to be fully elucidated. Marine bioluminescent genera (i.e. *Vibrio*, *Aliivibrio*, *Photobacterium,* and *Shewanella*) contain a large number of species which are both non-bioluminescent and closely associated with them. Additionally, it is thought that bioluminescent events may have evolved at least 40 times since their onset. Consequently, the isolation and identifica-

tion of new bioluminescent genera or species from different sources and determination of their genetic diversity are very important. There are different molecular typing methods to investigate the clonal relationships between bacteria. Pulsed-Field Gel Electrophoresis (PFGE) has the highest discriminatory power of all of them. The salient disadvantage of this technique accepted as the gold standard is that the results cannot be compared with each other due to the implementation of dissimilar protocols in different laboratories. In addition, PFGE results in classical protocols as long as three or four days (12). In PFGE, as a consequence of the cutting of the total genomic DNA by restriction endonucleases, PFGE fragment patterns are formed. For a large number of bioluminescent (3, 9, 13-15) and non-bioluminescent bacteria (16), this technique has been used with different restriction enzymes.

2. Objectives

To the best of our knowledge, the existing literature contains no PFGE data performed with the *NotI*-HF™ restriction enzyme with different bioluminescent bacteria. Thus, in this study, we aimed to determine the genetic relatedness of 10 different bioluminescent species isolated and identified from various marine sources. The data obtained from this study could lead to a better understanding of the bioluminescence mechanism.

3. Materials and Methods

3.1. Bioluminescent Strains

Forty-one bioluminescent strains isolated from different seawater, sediment, and fish samples and collected from regions at discrete depths in the Gulf of Izmir were used for this study. The bioluminescent bacteria identified by phenotypic and molecular methods (9, 15, 17) were streaked onto a Seawater Complete Agar (SWC) medium to obtain a single colony. After checking the purity of the strains, they were grown in a liquid SWC medium (18).

3.2. Preparation of Bioluminescent Strains

Bacterial suspensions with turbidity equivalent to Mc-Farland 1.0 were prepared in 200 µL HST buffer (cell suspension buffer) (10 mM Tris-HCl (Sigma-Aldrich, USA), 50 mM EDTA (Sigma-Aldrich, USA), and 20 mM NaCl (Sigma-Aldrich, USA)), and bacterial pellets were obtained by centrifuging at 13.000 grams for 2 minutes at 4°C. After discarding the supernatants, the pellets were suspended in 200 µL cold HST buffer (12).

3.3. Embedding of Strains into Agarose Plugs

Two percent low-melting agarose (Bio-Rad, USA) was prepared in the HST buffer. The agarose-buffer mixture was supplemented with 0.5 mL SDS (sodium dodecyl sulfate, Sigma-Aldrich; USA) (10 g/50 mL). Afterward, it was melted by heating in a magnetic stirrer at 50°C. The agarose mould (10 mm × 5 mm × 1.5 mm, Bio-Rad, USA) was kept at 4°C. Thereafter, 1.5 mL tubes for each bioluminescent strain were placed into a dry heat block at 50°C and 100 µL of an agarose-SDS mixture. Then, 100 µL of the bacteria suspension in the HST buffer was transferred into them. Subsequently, they were transferred into the agarose moulds without air bubbles and put at 4°C for hardening for 10 minutes (12).

3.4. Fractionation of Bacterial Cell into Agarose

Cell lysis solution I (50 mM Tris-HCl, 50 mM EDTA, 2.5 mg/mL lysosime (Sigma-Aldrich, USA) and 1.5 mg/mL proteinase K (Sigma-Aldrich, USA)) was prepared freshly. Agarose plugs with bacteria were removed from the moulds and placed into the lysis solution. All the tubes were incubated in a water bath at 37°C for 1 hour. After removing the cell lysis solution I, 600 µL of cell lysis solution II was prepared freshly (50 mM EDTA, 1% sarcosyl (Sigma-Aldrich, USA), and 400 µg/mL proteinase K) and was added into the tubes, and they were incubated in a water bath at 55°C for 1 hour (12).

3.5. Washing of Agarose Plugs after Cell Lysis

For solidification, 1.5 mL tubes in a water bath (55°C) were kept in ice for 15 minutes. Then, 5 - 15 mL of sterile ultra-pure water was transferred into each 50 mL of falcon tubes. Afterward, lysis solution II was aspirated and the agarose plugs were transferred carefully with a spatula into these falcon tubes and were incubated in a shaking water bath at 50°C for 15 minutes. Ultra-pure water was fully aspirated. Thereafter, 15 mL of tris-EDTA buffer was transferred into falcon tubes and they were incubated at 50°C with shaking at 15 minutes. This washing step was performed thrice (12).

3.6. Cutting the Whole DNA with the NotI-HF™ Restriction Enzyme in the Agarose Plugs

The agarose plugs containing DNA were cut in the ratio of 1/4 and were placed into a 100 µL fresh enzyme buffer at 37°C for 10 minutes. Next, 100 µL of 1Xbuffer solution was prepared with 100 µm/mL bovine serum albumin (Sigma-Aldrich, USA) and 20 units of fast digest *NotI*-HF™ (BioLabs 20,000 U/mL, England) and the genomic DNA embedded in agarose plugs were digested with *the NotI*-HF™ at 37°C for 30 minutes. After incubation, the tubes were held at 4°C for 15 minutes (12).

3.7. Loading the Agarose Plugs into Gel and Electrophoresis

High molar mass restriction fragments were observed by electrophoresis using 1% (w/v) pulsed-field grade agarose (Bio-Rad, USA) prepared with 0.5 X TBE in a CHEF-Mapper PFGE system (Bio-Rad, USA). The separation of the restriction fragments was carried out following running conditions: 6 V/cm² via 120° angle of impact for 24 hours at 14°C with 5 - 40 seconds pulse times. The gel was stained with

ethidium bromide (5 μg/mL, Sigma-Aldrich, USA) and then destained with sterile deionized water (12, 15). The band profile of the bioluminescent strains for *the NotI*-HF™ restriction enzyme was analyzed using Bio-Profil-1D++ software (Vilber Lourmat, Deutschland) at 10% homology coefficient. Dendrograms belonging to the PFGE band pro-

files were generated based on the Unweighted Pair-Group Method with Arithmetic Mean (UPGMA) (Vilber Lourmat).

4. Results

Table 1 shows the 10 different bioluminescent strains used in the present study (9).

Table 1. Bioluminescent Strains Used in the Present Study and their Accession Numbers together with the Sources, Depths, and Coordinates of the Sampling Dates

Isolation Date of Strains	Isolate	Accession Number	Source	Coordinate	Depth, m
30 March 2007					0 - 15
V. gigantis	SW15	JF412215	Seawater	38°29'03" N – 26°47'05"E	
V. gigantis	SWLiman	JF 412216	Seawater	38°27'22" N – 27°09'65"E	
A. fischeri	SW22	JF412240	Seawater	38°25'30" N – 26°58'60"E	
V. gigantis	SeLu25	JF412217	Sediment	38°23'50" N – 26°39'00"E	
22 January 2008					0 - 15
A. fischeri	S2W23	JF412241	Seawater	38°25'09" N – 27°02'86"E	
V. gigantis	S2W42	JF412218	Seawater	38°24'58" N – 26°56'88"E	
V. gigantis	S2W9	JF412219	Seawater	38°34'99" N – 26°39'00"E	
A. logei	Se2Lu45-2	JF412238	Sediment	38°25'03" N – 27°06'09"E	
V. gigantis	Se2Lu48	JF412223	Sediment	38°24'75" N – 26°58'90"E	
17 April 2008					0 - 15
V. gigantis	S3W46	JF412220	Seawater	38°26'70" N – 27°06'10"E	
V. gigantis	S3W28	JF412221	Seawater	38°23'50" N – 26°55'00"E	
V. gigantis	S3W2	JF412222	Seawater	38°40'90" N – 26°34'90"E	
V. gigantis	Se3Lu25	JF412224	Sediment	38°23'50" N – 26°39'00"E	
06 August 2008					0 - 15
V. harveyi	Se4Lu24	JF412244	Sediment	38°26'22" N – 27°04'68"E	
V. orientalis	Se4Lu49-2	JF412251	Sediment	38°24'35" N – 26°58'20"E	
V. azureus	Se4Lu15	JF412235	Sediment	38°29'03" N – 26°47'05"E	
30 March 2007				Between 38°37'00"N – 26°42'20"E and 38°37'45"N – 26°43'30"E	67
V. gigantis	FU-10	JF412225	*Mullus barbatus* (internal area)		
V. gigantis	FU-9	JF412226	*Diplodus annularis* (gill)		
V. lentus	FU-7	JF461265	*Alloteuthis subulata* (internal area)		
30 March 2007					50
A. logei	U-6	JF412239	*Merluccius merluccius* (intestine content)	Between 38°32'30" N – 26°45'50"E and 38°31'45"N – 26°45'20"E	
P. kishitanii	X-8	JF412253	*Zeus faber* (surface)	Between 38°26'50"N – 26°41'00"E and 38°26'00"N – 26°39'45"E	
S. woodyi	X-7	JF412255	*Pagellus erythrinus* (internal area)		
17 April 2008				Between 38°34'40"N – 26°46'10"E and 38°33'45"N-26°46'55"E	42 - 44
V. gigantis	E-14	JF412227	*Lepidotrigla cavillone* (gill)		
V. gigantis	E-16	JF412228	*Boops boops* (surface)		
V. gigantis	E-15	JF412229	*D. annularis* (surface)		
V. gigantis	E-10	JF412230	*Citharus linguatula* (gill)		
V. gigantis	E-11	JF412231	*Arnoglossus laterna* (internal area)		
A. fischeri	E-4	JF412242	*P. erythrinus* (gill)		
06 August 2008				Between 38°34'40"N – 26°46'10"E and 38°33'45"N-26°46'55"E	42 - 44
V. harveyi	H-15	JF412245	*C. linguatula* (internal area)		
V. harveyi	H-5	JF412246	*Scorpaena notata* (internal area)		
V. azureus	H-1	JF412236	*D. vulgaris* (gill)		
V. azureus	H-14	JF412237	*Trisopterus minutuscapelanus* (surface)		
V. harveyi	H-11	JF412247	*P. bogaraveo* (surface)		
V. crassostreae	H-7	JF412248	*A. laterna* (gill)		
V. gigantis	H-3	JF412232	*M. merluccius* (surface)		
V. crassostreae	H-12	JF412249	*Engraulis encrasicolus* (gill)		
V. crassostreae	H-19	JF412250	*Conger conger* (internal area)		
A. fischeri	H-18	JF412243	*Dentex macrophthalmus* (internal area)		
V. gigantis	H-2	JF412233	*D. annularis* (intestine contents)		
V. gigantis	H-16	JF412234	*B. boops* (gill)		
V. orientalis	H-9	JF412252	*L. cavillone* (gill)		

Each different group was evaluated separately in terms of PFGE fragment patterns. Three groups were composed (i.e. *V. harveyi, V. gigantis,* and other species). As is seen in Figure 1, all the *V. harveyi* strains could be typed with PFGE performed with the *NotI*-HF™ restriction enzyme (Figure 1 C). As a result

of the *NotI*-HF™ digestion, 2 main clusters (A and B) with 73% pattern homology were obtained. The closest strains with respect to genetic resemblance were achieved (i.e. *Se4Lu24 and H-15* strains) with 83% homology. The other strains (i.e. *H-5* and *H-11*) demonstrated 80% pattern homology (Figure 2).

Figure 1. Gel Images of the Forty-One Bioluminescent Strains Obtained From the Pulsed-Field Gel Electrophoresis Analysis With the NotI-HF™ Restriction Enzyme
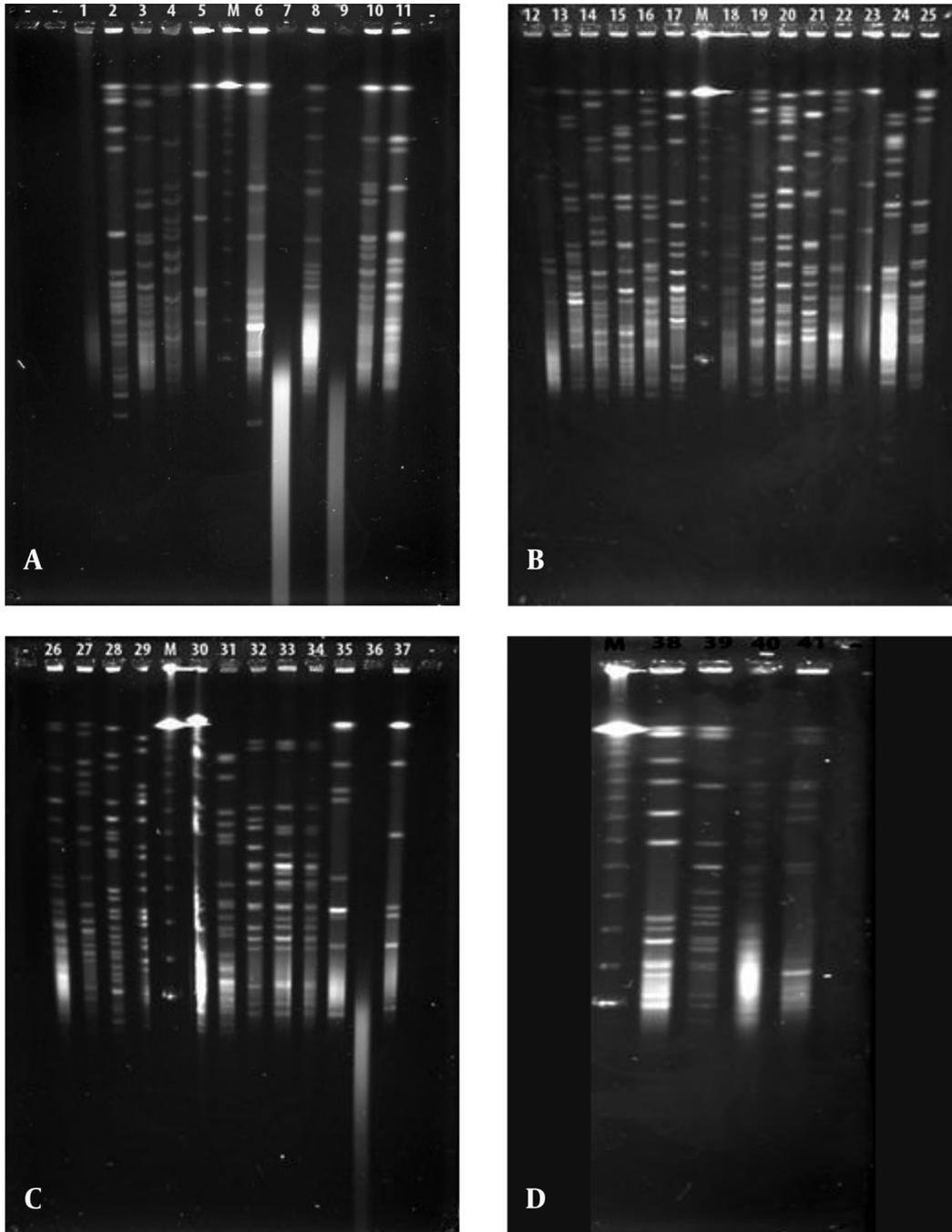
A, *V. gigantis* (1:E-16, 2:E-10, 3:E-11, 4:H-3, 7:S3W46, 8:S3W28, 9:H-16, 10:E-15, 11:H-2, 25:E-14), *A. logei* (5:Se2Lu45-2), and *A. fischeri* (6:S2W23) strains; B, *V. gigantis* (12:SW15, 13:SWLiman , 14:SeLu25 , 15:S2W42 , 16:S2W9 , 17:S3W2, 18:Se2Lu48 , 19:Se3Lu25, 20:FU-10, 21:FU-9), *V. lentus* (22:FU-7), *A. logei* (23:U-6), and *S. woodyi* (24:X-7) strains; C, *V. harveyi* (26:Se4Lu24, 27:H-15, 28:H-5, 29:H-11), *V. orientalis* (30:Se4Lu49-2, 31:H-9), *V. crassostreae* (32:H-7, 33:H-12, 34:H-19), and *A. fischeri* (35:SW22, 36:E-4, 37:H-18); and D, *V. azureus* (38:Se4Lu15, 39:H-1, 40:H-14) and *P. kishitanii* (41:X-8), M: Marker (Lambda Leader, Bio-Rad, USA, 48,5 kb - 1,000 kb).

Although 20 *V. gigantis* strains were used in this study, only 17 strains were typed with PFGE performed with the *NotI*-HF™ restriction enzyme. As a result of the PFGE analysis for the *V. gigantis* strains, 17 different PFGE fragment patterns were obtained (Figure 1 A and B). PFGE with the *NotI*-HF™ restriction enzyme resulted in 2 main clusters (A and B) and 17 different PFGE fragment patterns. *V. gigantis* strains *S3W46*, *E-16*, and *H-16* could not be typed. Clusters A and B exhibited 66% pattern homology. Cluster A consisted of two subclusters (A1 and A2), which displayed 68% pattern homology, with 16 different PFGE patterns that showed pattern homology between themselves ranging from 68 to 92%. In all the *V. gigantis* strains, the unrelated strain was *SWLiman* with 66% pattern homology. It was determined that the closest strains in terms of PFGE fragment patterns were observed between the *SeLu25* and *Se2Lu48* strains isolated from sediment and *S2W9* and *Se3Lu25* with 92% homology. In subcluster A2, 3 different groups (I, II, and III) were formed with 73% pattern homology. Thirteen different band patterns were

achieved in group I with pattern homology between 81% and 92% (Figure 3).
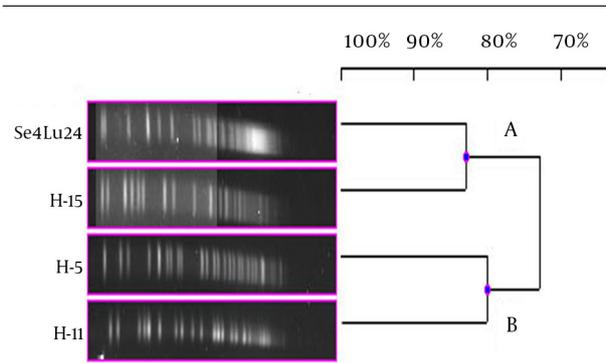
Figure 2. Dendrogram, Showing the Genetic Relationships Between the Bioluminescent *V. harveyi* Strains Based on the Pulsed-Field Gel Electrophoresis Analysis of the Genomic Restriction Fragments Formed With the *NotI*-HF™
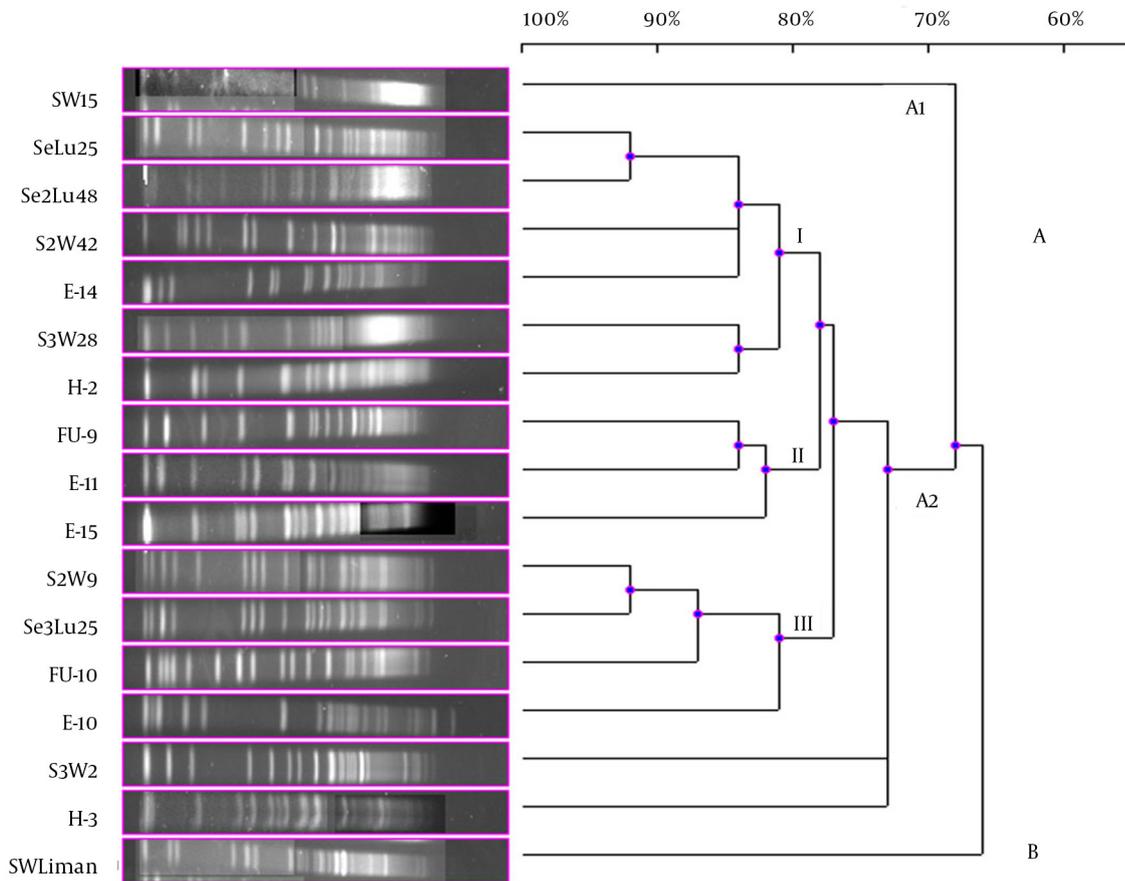
Figure 3. Dendrogram, Showing the Genetic Relationships Between the Bioluminescent *V. gigantis* Strains Based on the Pulsed-Field Gel Electrophoresis Analysis of the Genomic Restriction Fragments Formed With the *NotI*-HF™
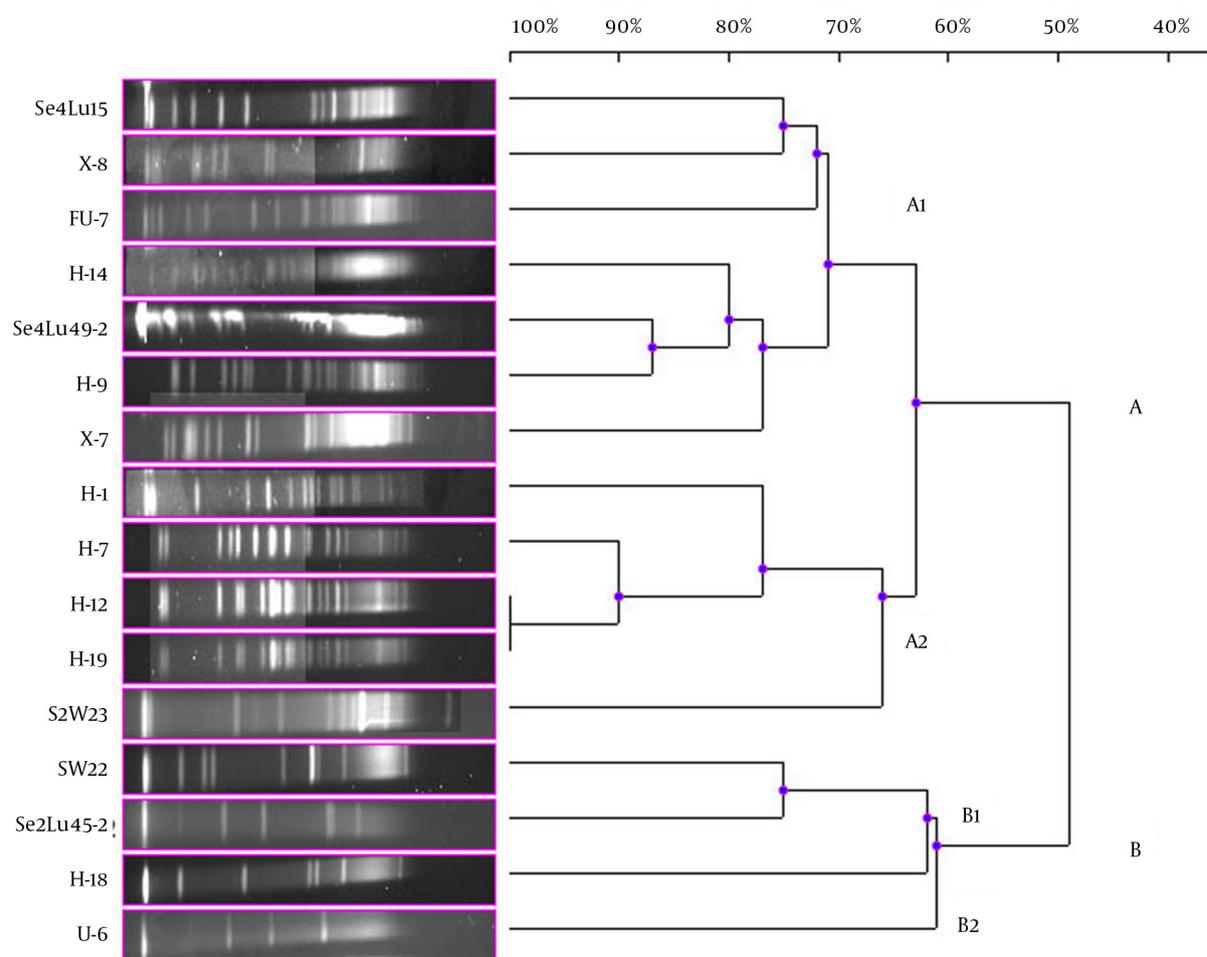
Figure 4. Dendrogram, Showing the Genetic Relationships Between the Bioluminescent *V. lentus*, *V. azureus*, *V. orientalis*, *S. woodyi*, *V. crassostreae*, *A. fischeri*, *A. logei,* and *P. kishitanii* Strains Based on the Pulsed-Field Gel Electrophoresis Analysis of the Genomic Restriction Fragments Formed with the *NotI*-HF™

In the current study, 41 bioluminescent strains isolated and identified were used and the remaining 17 strains were evaluated together from the point of PFGE fragment patterns with the *NotI*-HF™. However, only 16 strains were typed by this technique (Figure 1). The PFGE band pattern for the *A. fischeri* strain *E-4* could not be achieved. In this group, there were 3 *V. azureus* strains, 3 *V. crassostreae* strains, 2 *V. orientalis* strains, 3 *A. fischeri* strains, 2 *A. logei* strains, 1 *P. kishitanii strain*, 1 *V. lentus strain,* and 1 *S. woodyi* strain. In consequence of the *NotI*-HF™ digestion of the total genomic DNA, 2 main clusters (A and B) formed with 49% pattern homology. Considering all the bioluminescent strains in this group, PFGE fragment patterns with genome homology between 61% and 100% were achieved. Two *V. crassostreae* strains (i.e. *H-12 and H-9*) exhibited genome homology with the highest percentage (100%). Also, it was determined that the other *V. crassostreae* strain (i.e. *H-7*) was similar to the *H-12* and *H-9* strains at a ratio of 90% pattern homology. While the *V. orientalis* strains *Se4Lu49-2* and *H-9* had 87% genome homology, the *A. fischeri* (*S2W23,*

SW22, and H-18) and *A. logei* (*Se2Lu45-2* and *U-6*) strains between each other showed the lowest homology (61% and 62%). With the exception of *S2W23*, all the *A.* strains were located in cluster A (Figure 4).

5. Discussion

Within the scope of our study, the genetic diversity of 41 bioluminescent strains was determined. Ten different bioluminescent species were evaluated in terms of genomic polymorphism based on PFGE with the *NotI*-HF™ restriction enzyme. Moreover, so far, there have been no data indicating that the *V. lentus* and *V. crassostreae* strains are bioluminescent. It is, therefore, believed that these data will contribute to a better understanding of the evolution of bioluminescence. There are a large number of techniques for the characterization of bacterial species and also the evaluation of DNA similarity between two bacterial strains. The first definition method was DNA-DNA hybridization, but it is not suitable for the routine identification or determination of the genetic polymor-

phism of isolates because, although it is necessary to exhibit new bacterial genera and species, this technique is complex and limited to a few laboratories and also there is a dearth of relevant information (19).

To investigate clonal diversity and relationship between bacteria, PFGE with the highest discriminatory power has been used (12). PFGE is considered the gold standard because the structural integrity of the chromosome of the bacteria embedded in agarose plugs is not destroyed and the whole genome is profiled via cutting with the restriction enzyme. It has been proven that PFGE genomic DNA analysis is more discriminative in exhibiting phylogenetic diversity than analyses based on phenotype characterization (20). The genetic diversity and genotyping of some bioluminescent *Vibrio* isolates were determined by cutting their whole genome with 5 different restriction enzymes (i.e. *ApaI*, *EagI*, *NotI*-HF™, *SpeI*, and *SmaI*). DNA profiling with PFGE was introduced by Suwanto et al. in 1998 (13). In addition, some other studies have revealed further data relating to the PFGE analysis of bioluminescent bacteria (3, 21). In the present study, we determined clonal diversity and relationship using PFGE among bioluminescent *S. woodyi* strains (15) and *V. gigantis* strains (9). Nonetheless, to the best of our knowledge, the existing literature lacks research on the determination of genetic diversity via PFGE among bioluminescent *A. fischeri*, *A. logei*, *V. azureus*, *V. orientalis*, *V. lentus*, and *V. crassostreae* strains. The current study is, therefore, the first of its kind to report this genetic diversity. It is also deserving of note that the PFGE analysis of the *V. gigantis* strains with the *NotI*-HF™ has been carried out for the first time in the present study. The findings are very significant since *V. gigantis* was first reported as a bioluminescent strain in one of our previous studies (9). For assays, the *NotI*-HF™ was selected in light of a study performed by Suwanto et al. (13). The selection of the restriction enzyme is a critical variable in the PFGE process. Clonal diversity and relationship among the isolates are determined based on the restriction fragment band pattern. Accordingly, the fragments should be workable in number and distribution.

In the current study, 4 out of the 41 bioluminescent strains could not be typed by PFGE with the *NotI*-HF™ and although optimization assays were made and repeated three times for all the PFGE experiments, a smear appeared. Similarly, in a study performed by Eddabra et al. (21), 5 of the 30 *Vibrio* isolates could not be typed by PFGE with the *NotI*-HF™ due to the methylation of genomic DNA or DNA degradation during the process. In our study, because all the bioluminescent strains were isolated from different sources and depths, our findings reconfirm the heterogeneous structure of bioluminescent strains (21). In consequence of PFGE assays, 17 different band patterns were obtained as was expected. These differences obtained were not determined by phenotypic characterization. Whereas our previous study had determined that the bioluminescent *V. gigantis* strains had a few differences in their phenotypes, the findings of the

present study revealed that the *V. gigantis* strains had a high incidence of polymorphism when the whole genome was analyzed via PFGE with the *NotI*-HF™.

The highest percentage of genome similarity in the 17 *V. gigantis* strains used in the current experiment was found to be 92%. *SeLu25 and Se2Lu48* were two sediment strains isolated from different seasons and stations but from the same depth. Additionally, *S2W9* and *Se3Lu25* were seawater and sediment strains, respectively, and even though they were isolated from the same depth, their isolation sources were different. In contrast, the *SeLu25-Se2Lu48 (92%) and S2W9-Se3Lu25 (92%) strains were found to be genetically closer than H-2, FU-9, and E-15 (78%)* isolated from the same fish species. Although *SW15, SWLiman, and SeLu25* were isolated from the same depth in the same season, it was become evident that *SWLiman* was different from all other *V. gigantis* strains and it showed 66% genome similarity with the others. In addition, it was determined that only bioluminescent *V. gigantis* strains showed distribution in Diplodus annularis and they were not completely identical in terms of whole genome and also genotypic similarity among them was at a ratio of 78%.

As a result of the PFGE assays, all the *V. harveyi* strains were typed and 4 different PFGE band patterns were achieved. As well as being isolated in the same season; while sediment sample *Se4Lu24* was obtained from a depth of 0 - 15 meters, the other *V. harveyi* strains (i.e. *H-15, H-5, and H-11*) were taken from a depth of 42 - 44 meters. In consequence of the PFGE assay, these strains were divided into 2 main clusters with 73% of pattern homology. Contrary to expectation, the closest genome similarity (83%) was achieved from 2 strains, *Se4Lu24* (a sediment isolate) and *H-15* (isolated from *Citharus linguatula*). To determine why bioluminescence has come into being in the evolutionary process, some studies have been performed, especially on the *V. harveyi* strains, and significant results have been obtained (22). Hence, the determination of the genetic proximity of these strains to one another will contribute greatly to the understanding of these mechanisms and we hope that our findings will play a part in future investigations.

In the present study, the remaining 17 bioluminescent strains were examined together. *A. fischeri E-4* strain was not typed. On dendrogram consisting of the 2 main clusters, it was observed that the *Aliivibrio* strains were gathered in cluster B and the other bioluminescent strains were in cluster A. Then again, it was determined that the *Aliivibrio* strains were different from the other bioluminescent *Aliivibrio* and *Photobacterium* species and their genome similarity was 49%. Only the *A. fischeri S2W23* strain was located in cluster A and it showed 63% of genome similarity with these species in a different subcluster of cluster A. The *Aliivibrio* genus was incorporated in the *Vibrionaceae* family in 2007 in the sequel of a study performed by Urbanczyk et al. (1), who reported that some *Vibrio* species were different from the species in the *Vibrionaceae* family in terms of phylogenetic and phenotypic

features. These results chime in with the findings of our study.

A similar situation was observed in the bioluminescent strain *V. azureus*. The results showed that 3 *V. azureus* strains emerged in different subclusters in cluster A and each of them displayed higher genome homology with different bioluminescent strains than similarity among themselves. Contrary to this, a high PFGE band pattern resemblance was determined in the *V. orientalis* and *V. crassostreae* strains. Based on the band pattern, especially 2 *V. crassostreae* strains (i.e. *H-12* and *H-19*) were completely identical and the other *V. crassostreae* strain (i.e. *H-7)* exhibited 90% genome homology with these 2 strains. The *V. orientalis* strains *Se4Lu49-2 and H-9* were more similar to each other in terms of the genomic feature than the other species, and the homology of the band pattern was 87%.

The V. lentus, *P. kishitanii,* and *V. azureus* strains, which were first reported in the literature in 2001, 2007, and 2010, respectively, were located in the same subcluster in the present study. Our results showed that especially the new bioluminescent strains, i.e. *P. kishitanii* and *V. azureus*, had 75% of the PFGE band pattern homology. The *V. lentus* strains were isolated and identified for the first time in 2001 and characterized by various research groups in subsequent years (23, 24). However, none of these studies showed that this strain was bioluminescent. Our findings revealed that the *V. lentus* strains could also show bioluminescence and had genome similarity at a ratio of 75% with the bioluminescent species recently reported in the literature. In addition, the PFGE analysis demonstrated that the *V. lentus* strains emerged separately from the other bioluminescent strains in the different subclusters.

The PFGE genomic DNA analysis is more discriminative in revealing genetic diversity and polymorphism among related species than analyses based on phenotypic characterization. A study performed by Suwanto (20) revealed that 25 isolates characterized by physiological analysis came together under 2 *Vibrio* species. Moreover, when the PFGE analysis with the *NotI*-HF™ was performed, 13 different genotypes were ingenerated. The analyses of the phenotypic characterization of these strains have verified that the discriminatory power of PFGE is higher than that of the other techniques. Our results revealed that 37 bioluminescent strains belonged to 4 different genera and 10 different species had 36 different genotypes.

To our knowledge, there have not been any PFGE studies including 4 bioluminescent genera. The present study is, therefore, the first of its kind in terms of its scope. With the discovery of new bioluminescent species exhibiting new bioluminescent features, we hope that the findings of the present study will contribute to a better understanding of the mechanism and evolution of bioluminescence and polymorphism in different bioluminescent strains. The strains investigated in the current study were isolated and identified from different marine sources and consisted of the 4 bioluminescent genera identified so far. Moreover, in the present study, the PFGE assay was performed by using the *NotI*-HF™ restriction enzyme and the genetic diversity of 37 bioluminescent strains was demonstrated.

Acknowledgements

References

1. Urbanczyk H, Ast JC, Higgins MJ, Carson J, Dunlap PV. Reclassification of *Vibrio fischeri, Vibrio logei, Vibrio salmonicida* and *Vibrio wodanis* as *Aliivibrio fischeri* gen. nov., comb. nov., *Aliivibrio logei* comb. nov., *Aliivibrio salmonicida* comb. nov. and *Aliivibrio wodanis* comb. nov. *Int J Syst Evol Microbiol.* 2007;**57**(Pt 12):2823–9.
2. Kita-Tsukamoto K, Wada M, Yao K, Kamiya A, Yoshizawa S, Uchiyama N, et al. Rapid identification of marine bioluminescent bacteria by amplified 16S ribosomal RNA gene restriction analysis. *FEMS Microbiol Lett.* 2006;**256**(2):298–303.
3. Ast JC, Cleenwerck I, Engelbeen K, Urbanczyk H, Thompson FL, De Vos P, et al. *Photobacterium kishitanii* sp. nov., a luminous marine bacterium symbiotic with deep-sea fishes. *Int J Syst Evol Microbiol.* 2007;**57**(Pt 9):2073–8.
4. Yoshizawa S, Wada M, Kita-Tsukamoto K, Yokota A, Kogure K. *Photobacterium aquimaris* sp. nov., a luminous marine bacterium isolated from seawater. *Int J Syst Evol Microbiol.* 2009;**59**(Pt 6):1438–42.
5. Yoshizawa S, Wada M, Kita-Tsukamoto K, Ikemoto E, Yokota A, Kogure K. *Vibrio azureus* sp. nov., a luminous marine bacterium isolated from seawater. *Int J Syst Evol Microbiol.* 2009;**59**(Pt 7):1645–9.
6. Yoshizawa S, Wada M, Yokota A, Kogure K. *Vibrio sagamiensis* sp. nov., luminous marine bacteria isolated from sea water. *J Gen Appl Microbiol.* 2010;**56**(6):499–507.
7. Urbanczyk H, Ast JC, Kaeding AJ, Oliver JD, Dunlap PV. Phylogenetic analysis of the incidence of lux gene horizontal transfer in Vibrionaceae. *J Bacteriol.* 2008;**190**(10):3494–504.
8. Yoshizawa S, Karatani H, Wada M, Yokota A, Kogure K. *Aliivibrio sifiae* sp. nov., luminous marine bacteria isolated from seawater. *J Gen Appl Microbiol.* 2010;**56**(6):509–18.
9. *Ersoy Omeroglu E, Karaboz I. Characterization and genotyping by pulsed-field gel electrophoresis (PFGE) of the first bioluminescent Vibrio gigantis strains. Afr J Microbiol Res. 2012;6(43):7111–22.*
10. Le Roux F, Goubet A, Thompson FL, Faury N, Gay M, Swings J, et al. *Vibrio gigantis* sp. nov., isolated from the haemolymph of cultured oysters (*Crassostrea gigas*). *Int J Syst Evol Microbiol.* 2005;**55**(Pt 6):2251–5.
11. Stabili L, Giangrande A, Pizzolante G, Caruso G, Alifano P. Characterization of vibrios diversity in the mucus of the polychaete *Myxicola infundibulum* (Annellida, Polichaeta). *Microb Ecol.* 2014;**67**(1):186–94.
12. Durmaz R, Otlu B, Koksal F, Hosoglu S, Ozturk R, Ersoy Y, et al. The optimization of a rapid pulsed-field gel electrophoresis protocol for the typing of *Acinetobacter baumannii, Escherichia coli* and *Klebsiella* spp. *Jpn J Infect Dis.* 2009;**62**(5):372–7.
13. Suwanto A, Yuhana M, Herawaty E, Angka SL. Genetic diversity of luminous *Vibrio* isolated from shrimp larvae. In: Flegel TW, editor. *Advances in shrimp biotechnology*. Bangkok, Thailand: National Center for Genetic Engineering and Biotechnology; 1998. pp. 217–24.
14. Oh HS, Kum KA, Kim EC, Lee HJ, Choe KW, Oh MD. Outbreak of *Shewanella algae* and *Shewanella putrefaciens* infections caused by a shared measuring cup in a general surgery unit in Korea. *Infect Control Hosp Epidemiol.* 2008;**29**(8):742–8.
15. Ersoy Omeroglu E, Karaboz I, Sudagidan M. Characteristics and genetic diversity of bioluminescent *Shewanella woodyi* strains

isolated from the Gulf of Izmir, Turkey. *Folia Microbiol (Praha)*. 2014;**59**(1):79–92.

16. Cameron DN, Khambaty FM, Wachsmuth IK, Tauxe RV, Barrett TJ. Molecular characterization of *Vibrio cholerae* O1 strains by pulsed-field gel electrophoresis. *J Clin Microbiol*. 1994;**32**(7):1685–90.

17. Ersoy Omeroglu E. Isolation, phenotypic and molecular characterization of bioluminescent bacteria from Izmir Bay. Ege University, Doctoral thesis; 2011.

18. Liu PC, Chuang WH, Lee KK. Infectious gastroenteritis caused by *Vibrio harveyi* (*V. carchariae*) in cultured red drum, *Sciaenops ocellatus*. *J Appl Ichthyol*. 2003;**19**(1):59–61.

19. Gevers D, Cohan FM, Lawrence JG, Spratt BG, Coenye T, Feil EJ, et al. Opinion: Re-evaluating prokaryotic species. *Nat Rev Microbiol*. 2005;**3**(9):733–9.

20. Suwanto A. Genetic diversity of ampicillin-resistant *Vibrio* iso-lated from various stages of tiger shrimp larvae development. *Biotropia*. 2000;(15):36–47.

21. Eddabra R, Prevost G, Scheftel JM. Rapid discrimination of environmental *Vibrio* by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. *Microbiol Res*. 2012;**167**(4):226–30.

22. Czyz A, Plata K, Wegrzyn G. Stimulation of DNA repair as an evolutionary drive for bacterial luminescence. *Luminescence*. 2003;**18**(3):140–4.

23. Macian MC, Ludwig W, Aznar R, Grimont PA, Schleifer KH, Garay E, et al. *Vibrio lentus* sp. nov., isolated from Mediterranean oysters. *Int J Syst Evol Microbiol*. 2001;**51**(Pt 4):1449–56.

24. Farto R, Armada SP, Montes M, Guisande JA, Perez MJ, Nieto TP. Vibrio lentus associated with diseased wild octopus (*Octopus vulgaris*). *J Invertebrate Pathol*. 2003;**83**(2):149–56.