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Application of PCR-Based DNA Fingerprinting Techniques for the Genetic Differentiation of *Vibrio cholerae* Non-01/Non-0139 Isolates from Kuching, Sarawak

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ABSTRACT

Fifty-four *Vibrio cholerae* non-01/non-0139 isolates were evaluated for their genetic diversity via randomly amplified polymorphic DNA-PCR (RAPD-PCR), enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR) and BOX-PCR assays. Based on the various PCR fingerprints, the *V. cholerae* isolates were successfully differentiated into 39 types by RAPD-PCR, 43 types by ERIC-PCR, and 38 types by the BOX PCR, with the overall average polymorphic distances observed to be at 0.593, 0.527 and 0.504, respectively. The Simpson's index of diversity of the isolates based on the fingerprinting analyses indicated that these DNA fingerprinting methods have high discriminatory power 0.986 (RAPD-PCR), 0.992 (ERIC-PCR) and 0.983 (BOX-PCR), and could be used independently or as supplements to other methods for the epidemiological investigation of the *V. cholerae* from water and seafood sources. The dendrograms constructed also showed that the *V. cholerae* isolates were clustered into several main clusters and sub-clusters, suggesting that different strains were circulating in the water environment and in the seafood sources. We conclude that molecular genotyping of *V. cholerae* isolates from surface water and seafood samples in Kuching, Sarawak (Malaysia) enabled high level observation of clonal diversity within *V. cholerae* isolates, and is directly applicable for the molecular epidemiological studies of the *V. cholerae* isolates.

Keywords**:** *V. cholerae* non-01/non-0139, DNA fingerprinting, RAPD-PCR, ERIC-PCR, BOX-PCR

INTRODUCTION

Vibrio cholerae is a Gram-negative and motile curved rod bacterium that is ubiquitous in the environment (Banerjee *et al*., 2014; Broeck *et al*. 2007). This bacterium, like other *Vibrio spp*., represents a component of the autochthonous flora of brackish water, estuaries and salt marshes of coastal areas of the tropics and subtropics (Lesley *et al*., 2011; Lipp *et al.* 2002; Micky *et al*., 2014). *V. cholerae* is best known as the causative agent of cholera, causing morbidity and mortality in many areas of Asia, Africa and Latin America (Banerjee *et al*., 2014; Zhang *et al*., 2013). Previous studies have reported the outbreak of this endemic disease to be caused by two particular strains designated *V. cholerae* 01 or 0139 through the ingestion of seafood and water mainly via the faecal-oral route (Banerjee *et al*., 2014; Zhou *et al*., 2014).

Thus, there is possible risk of infection from the consumption of seafood that may be naturally contaminated by this bacterium since *V. cholerae* has the aquatic habitat as their natural niche (Tapchaisri *et al*. 2007; Zhou *et al*., 2014). Therefore, there is a need to constantly monitor the occurrence, the pathogenicity and the distribution of *V. cholerae* from environmental and food sources as it is essential for action during outbreaks and sporadic attacks.

The pathogenicity of *V. cholerae* 01 and 0139 strains is mainly associated with their ability to produce cholera toxins (CT), which is encoded in its "virulence cassette" region of the chromosome that consists of the cholera toxin A (*ctx*A*),* cholera toxin B *(ctx*B*),*accessory cholera enterotoxin *(ace)* and zonula occludens toxin (*zot)* genes (Fooladi *et al*., 2013; Morita

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et al., 2010; Son *et al.*, 2002). However, the epidemiological impact of environmental non-cholera causing *V. cholerae* non-01/non-0139 strains is not clearly understood, since the majority of environmental *V. cholerae* isolates do not contain toxin genes (>95%) and lack not only the virulence gene cassette for CT but also *zot* and *ace* (Bakhshi *et al*., 2009; Son *et al*., 2002; Tapchaisri *et al*., 2007). Hence, a study of the molecular diversity of naturally occurring *V. cholerae* non-01/non-0139 strains should offer insights into the ecology, evolution and epidemiology of *V. cholerae* non-01/non-0139 as a species.

In the past, the ability to differentiate individual strains of *V. cholerae* for epidemiological purposes has been hampered by the low discriminatory capability of available typing systems, such as biochemical and serologic identification. These approaches are laborious, time-consuming and insufficiently sensitive in identifying *V. cholerae* (Bhowmick *et al*., 2007). Therefore, in recent years, the genetic diversity and molecular epidemiology of *V. cholerae* O1 and non-O1 have been performed by using various molecular techniques such as Pulsed-Field Gel Electrophoresis (PFGE), Ribotyping, Multi-Locus Enzyme Electrophoresis (MLEE) and variants of polymerase chain reaction (PCR)-based methods (Jiang *et al*., 2000; Tapchaisri *et al*., 2007; Zhang *et al*., 2007; Zhou *et al*., 2014). PCR-based genotyping methods routinely used to type *V. cholerae* include enterobacterial repetitive intergenic consensus sequence-based PCR (ERIC-PCR), randomly amplified polymorphic DNA (RAPD-PCR) and BOX element PCR (Bhowmick *et al*., 2007; Rivera *et al*., 1995; Son *et al*., 2002). These methods are generally rapid and easy to execute and have been used in differentiating strains of V. cholerae O1 and non-O1 in epidemiological investigations, and also to study relatedness among bacteria strains in order to trace the origin and geographical distribution of *V. cholerae* (Rivera *et al.*, 1995; Son *et al*., 2002). These different PCR-based genotypic methods differ in their discriminatory degree. Thus, the objective of this study is to perform ERIC-PCR, RAPD-PCR and BOX-PCR to determine their discriminatory power in the differentiation of the *V. cholerae* from surface water and seafood samples.

MATERIALS & METHODS

Source of *Vibrio cholerae* Non 01/Non-0139 Isolates

All the *V. cholerae* non-01/non-0139 cultures used in this study were obtained from the Microbiology Laboratory UNIMAS culture collection, isolated from the period of December, 2003 to July, 2004 via standard procedures (Son *et al*., 2004) from seafood samples (*Anadara granosa*) and surface water within the Kuching-Samarahan district of Sarawak, East Malaysia (Figure 1). All 54 isolates were revived in Nutrient broth (Oxoid, UK) supplemented with 3% (w/v) NaCl and subjected to multiple standard biochemical testing for confirmation as described by Choopun *et al*. (2002). The list of the isolates is shown in Table 1.

Genomic DNA extraction

The total genomic DNA of the 54 *V. cholerae* isolates was extracted by using Wizard® Genomic DNA Purification Kit (Promega, USA) according to the manufacturer's instruction.

Randomly amplified polymorphic DNA-PCR (RAPD-PCR)

The RAPD-PCR was performed based on the protocol described by Son *et al.* (2002). Ten 10-mers primers with 50% GC content were previously screened for their ability to amplify the genome of representative *V. cholerae* isolates. Based on the preliminary results obtained, primer GEN-01-50-09 (Table 2) was selected for further RAPD-PCR analysis of all the *V. cholerae* isolates as this primer provided the best banding patterns. The PCR amplification reaction consisted of 25 µl volume containing 1 µl of 20-30 ng of genomic DNA, 0.5 µl each of 5 pmol primer, 2.5 µl of 10X PCR buffer, 3.0 µl of 25 mM $MgCl_2$, 0.5 µl of 10 mM dNTPs, and 0.5 µl *Taq* DNA polymerase (5 U/µl) and 16.5 µl of sterile distilled water. Amplifications were carried out in a thermocycler (Perkin Elmer 2400, USA) as follows: 94ºC for 3 min and 45 cycles of denaturation at 94ºC for 1 min, annealing at 33ºC for 1 min, and an extension at 72ºC for 2 min. A final elongation step at 72ºC for 5 min

were included at the end of the 45 cycles.

Enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR)

Enterobacterial Repetitive Intergenic Consensus-PCR (ERIC-PCR) was carried out as described by Rivera *et al*. (1995). A pair of oligonucleotide primers, ERIC-1R and ERIC-2 (Table 1), was used. The following was added to each of the 25 µl PCR mixture: 2.5 µl of 10X PCR buffer, 2.5 µl of 25 mM MgCl2, 0.5 µl of 10 mM dNTPs mixture, 1 µl of 5 pmol each of the ERIC-1R and ERIC-2 primers, 1.0 µl of template DNA, 0.5 µl of Taq DNA polymerase (5 U/ µl) and 16.0 µl of sterile distilled water. Amplifications were carried out in a thermocycler (Perkin Elmer 2400, USA) under the following conditions: 95ºC for 5 min and 35 cycles of denaturation at 92ºC for 1 min, annealing at 52ºC for 1 min, and an extension at 72ºC for 3 min. A final elongation step at 72ºC for 10 min were included at the end of the 35 cycles.

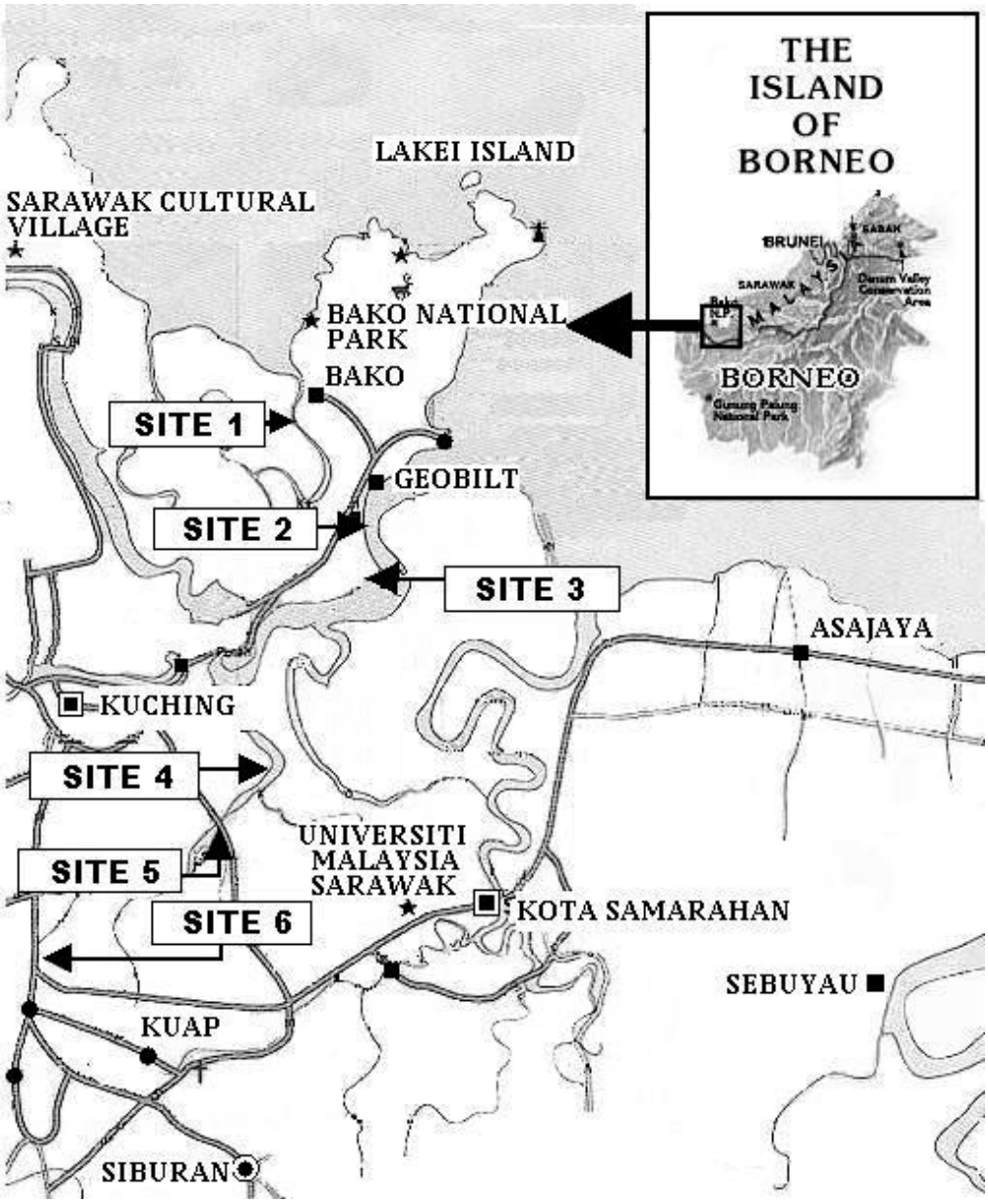

Figure 1. Locations of sampling site. (Site 1 – Sg. Bako, Site 2 – Sg. Sejingkat, Site 3 – Tk. Sejingkat, Site 4 – Sg. Tabuan, Site 5 – Sg. Kuap, Site 6 - Padawan)

Table 1. The list of the 54 *V. cholerae* non-01/non-0139 strains isolated from surface water and seafood samples in Kuching, Sarawak (Malaysia).

Isolate	Month of sampling	Location	Source
DC1	Dec, 2003	Sg. Sejingkat	SW
DC2	Dec, 2003	Sg. Sejingkat	SW
DC3	Dec, 2003	Sg. Sejingkat	SW
DC4	Dec, 2003	Sg. Tabuan	SW
DC5	Dec, 2003	Sg. Tabuan	SW
DC6	Dec, 2003	Sg. Tabuan	SW
DC7	Dec, 2003	Sg. Tabuan	SW
DC8	Dec, 2003	Sg. Tabuan	SW
DC9	Dec, 2003	Sg. Tabuan	SW
DC10	Dec,2003	Padawan	SF
DC11	Dec,2003	Padawan	SF
JC1	Jan, 2004	Tk. Sejingkat	SW
JC2	Jan, 2004	Tk. Sejingkat	SW
JC3	Jan, 2004	Sg. Tabuan	SW
FC1	Feb, 2004	Sg. Bako	SW
FC2	Feb, 2004	Sg. Bako	SW
FC3	Feb, 2004	Sg. Bako	SW
FC4	Feb, 2004	Sg. Bako	SW
FC5	Feb, 2004	Sg. Bako	SW
FC6	Feb, 2004	Sg. Sejingkat	SW
FC7	Feb, 2004	Sg. Sejingkat	SW
FC8	Feb, 2004	Sg. Sejingkat	SW
FC9	Feb, 2004	Tk. Sejingkat	SW
FC10	Feb, 2004	Tk. Sejingkat	SW
FC11	Feb, 2004	Tk. Sejingkat	SW
FC12	Feb, 2004	Sg. Tabuan	SW
FC13	Feb, 2004	Sg. Tabuan	SW
FC14	Feb, 2004	Sg. Tabuan	SW
FC15	Feb, 2004	Sg. Tabuan	SW
FC16	Feb, 2004	Sg. Tabuan	SW
FC17	Feb, 2004	Sg. Kuap	SW
FC18	Feb, 2004	Sg. Kuap	SW
MC1	Mac, 2004	Sg. Bako	SW
MC2	Mac, 2004	Sg. Bako	SW
MC3	Mac, 2004	Sg. Bako	SW
MC4	Mac, 2004	Sg. Tabuan	SW
MC5	Mac, 2004	Sg. Tabuan	SW
MC6	Mac, 2004	Sg. Tabuan	SW
MC7	Mac, 2004	Sg. Tabuan	SW
MC8	Mac, 2004	Sg. Tabuan	SW
MC9	Mac, 2004	Tk. Sejingkat	SW
MC10	Mac, 2004	Tk. Sejingkat	SW
MC11	Mac, 2004	Tk. Sejingkat	SW
MC12	Mac, 2004	Sg. Sejingkat	SW
MC13	Mac, 2004	Sg. Sejingkat	SW
MC14	Mac, 2004	Sg. Sejingkat	SW
AC1	Apr, 2004	Tk. Sejingkat	SW
AC2	Apr, 2004	Tk. Sejingkat	SW
AC3	Apr, 2004	Tk. Sejingkat	SW
AC4	Apr, 2004	Tk. Sejingkat	SW
AC5	Apr, 2004	Tk. Sejingkat	SW
AC6	Apr, 2004	Tk. Sejingkat	SW
AC7	Apr, 2004	Tk. Sejingkat	SW
AC8	Apr, 2004	Sg. Kuap	SW

Note : SW - Surface Water; SF - Seafood

Table 2. List of primers used for the PCR amplification.

Primer	Primer sequences	Tm (^{o}C)	Reference
GEN-01-50-09	5'-AGAAGGGATG–3'	33	Son *et al*., 2002
ERIC-1R	5' -ATG TAA GCT CCT GGG GAT TCA C- 3'	52	Velsalovic *et al*., 1991
ERIC-2	5' -AAG TAA GTG ACT GGG GTG AGC G- 3'		
BOXA1R	5'-CTACGGCAAGGCGACGCTGACG-3'	43	Rivera *et al*., 1995

BOX-PCR

BOX-PCR was performed using the primer BOXA1R (Table 1). The PCR amplification reactions consisted of 25 µl volume containing 1.0 µl of 20-30 ng of template DNA, 2.5 µl of 10X PCR buffer, 2.5 µl of 25 mM $MgCl_2$, 0.5 µl of *Taq* DNA polymerase (5 U/µl), 0.5 µl of 10 mM of dNTP mixture, 17.0 µl of sterile distilled water and 1 µl of 5 pmol of primer. The amplification protocol was as follows: initial denaturation at 95°C for 2 min; 35 cycles of 94°C for 3 min, 92°C for 30 s, 43°C for 1 min, and 65°C for 3 min; and final extension at 65°C for 8 min. The amplification was carried out in a Perkin Elmer 2400 (USA) thermocycler.

Agarose Gel Electrophoresis And Gel Staining

The amplification products were electrophoresed in a 1.2% (w/v) agarose in 1X TBE (0.1 M Tris, 0.1 M Boric acid, 0.1 mM EDTA) at 90 V for 40 min. Gels were stained in 0.5 µg/ml ethidium bromide for 15 minutes and was destained in distilled water for 30 min before viewing under ultraviolet illumination. The gel was examined and the images were captured under UV light transillumination with gel documentation system (Bio-Rad, USA).

Data analysis

The fingerprint profiles obtained from RAPD-PCR, ERIC-PCR and BOX-PCR were analyzed using the RAPDistance Package Version 1.04 (Armstrong *et al.,* 1996). The banding patterns obtained from the gel were scored in the binary data format for each of the primers used. The scoring was made based on the presence ('1') or absence ('0') of the band. The distance calculation of similarity among the band profiles were performed based on the Dice (Nei & Li, 1979) formulation via the RAPDistance Software Package. The distance matrix value, D was calculated with the following formula:

$$D = 1 - \frac{2n_{xy}}{2n_{xy}+n_{01}+n_{10}}$$

n_{xy} = the number of shared bands (e.g. x and y)

n_{01} = the number of band position where x=0 and y=1.

n_{10}= the number of band position where x=1, and y=0.

From the calculation of the Dice formulation, a dendrogram of NJTree (Neighbour-joining tree) format was generated by means of the TDraw clustering algorithm program (Li & Ferguson, 1998).

Simpson's Index of Diversity was then used as a method for deriving a numerical index of the discriminatory ability of the RAPD-PCR, ERIC-PCR and BOX-PCR assays. The discriminative ability of these PCR-based methods can be evaluated through the values derived from calculation, which are presented as the discrimination index (Hunter & Gaston, 1988).

The Simpson Index of Diversity was calculated based on formula below:

Simpson's Index of Diversity = 1 – S, where S = Simpson's Index.

$$\text{Simpson's Index, } S = \frac{\sum n(n-1)}{N(N-1)}$$

n = the total number of isolates of a particular banding pattern

N = the total number of isolates in this study

RESULTS

V. cholerae non-01/non-0139 in surface water and seafood samples

A total of 54 *V. cholerae* non-01/non-0139 strains were successfully revived from the surface water (n=52) and seafood samples (n=2) throughout the sampling period.

RAPD-PCR Profiles

The representative profile of the RAPD-PCR patterns obtained with primer GEN-01-50-09 is shown in Figure 2. The RAPD profiles showed a variety of patterns with a total number of 39 different patterns. Based on the dendrogram (Figure 3) generated from the RAPD-PCR patterns, the isolates were clustered into two main clusters (Clusters 1 and 2) and these main clusters were further subdivided into several subclusters, defining the genetic heterogeneity among the isolates. Based on the dendrogram, 46 of the 54 *V. cholerae* isolates are in Cluster 2 and the remaining eight isolates are grouped in Cluster 1. Isolates in Cluster 1 originated from seafood (shrimp) and water samples from Tambak Sejingkat, whereas the remaining water samples isolates were grouped into Cluster 2. Cluster 2 is further subdivided into four subclusters (2A, 2B, 2C, 2D), with average genetic distance, D = 0.577, indicating 42.3% similarity between all of 54 isolates tested. Cluster 1 is more homogenous, with a lower average genetic distance, D of 0.405 (59.5% similarity). The average genetic distance between individual isolates was D = 0.593, indicating 40.7% of overall similarity between all of the 54 strains.

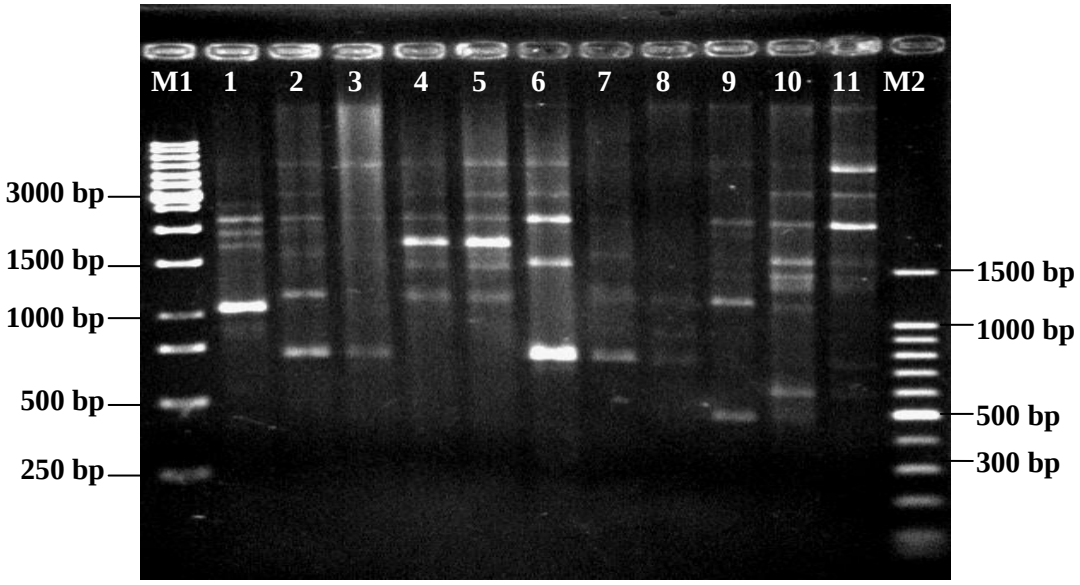

Figure 2. Agarose gel (1.2% w/v) electrophoresis of RAPD-PCR fingerprints of the *V. cholerae* representative isolates obtained with primer GEN-01-50-09. M1: 1 kb DNA ladder. Lane 1-8: DC1-DC11. M2: 100 bp DNA ladder.

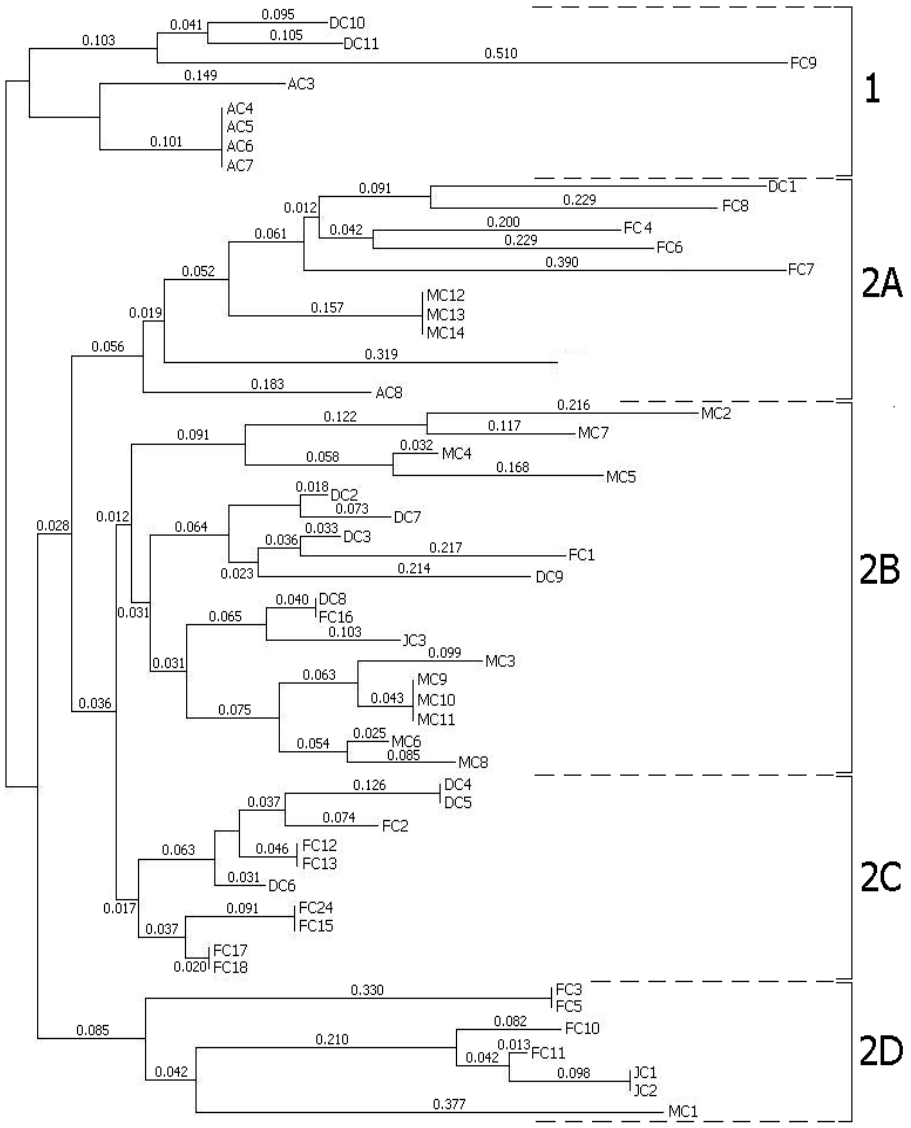

Figure 3. Dendrogram of the 54 *V. cholerae* isolates constructed using the RAPD-PCR data as presented in the neighbor joining tree (NJTree) format. Numbers represent distance value between the isolates.

ERIC- PCR Profiles

Figure 4 shows the representative profile of the ERIC-PCR patterns obtained using the ERIC primers. Using this primer, the 54 *V. cholerae* isolates also showed high degree of polymorphism with a total of 43 ERIC-PCR patterns generated, each characterized by between five to 16 bands. Dendrogram (Figure 5) constructed from the ERIC-PCR profiles generated two major clusters consisting of 34 and 20 isolates, respectively, with an average distance of 0.527. From the ERIC-PCR assay, the average distance for the two clusters is almost the same, with the value of D = 0.416 and 0.482, respectively, indicating 58.4% and 51.8% of similarity among the isolates within the clusters with the greatest genetic distance observed being D = 0.77.

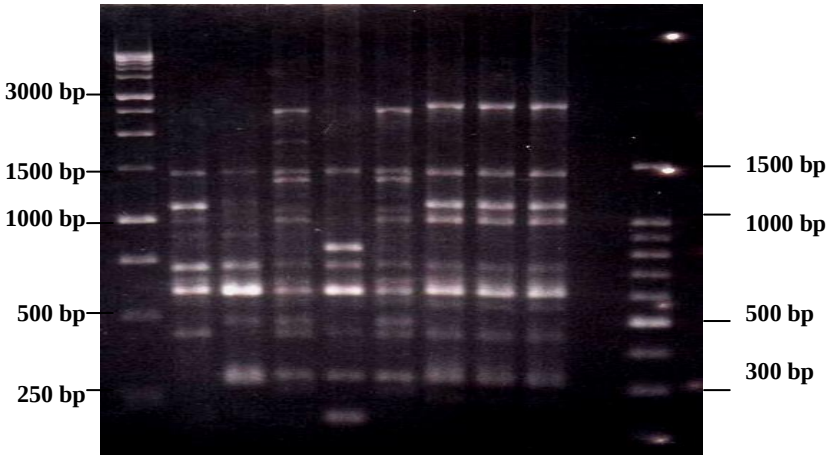

Figure 4. Agarose gel (1.2% w/v) electrophoresis of ERIC-PCR fingerprints of the *V. cholerae* representative isolates obtained with primers ERIC1R and ERIC2. Lane 1-8: FC1-FC8, N: negative control, M2: 100 bp DNA ladder.

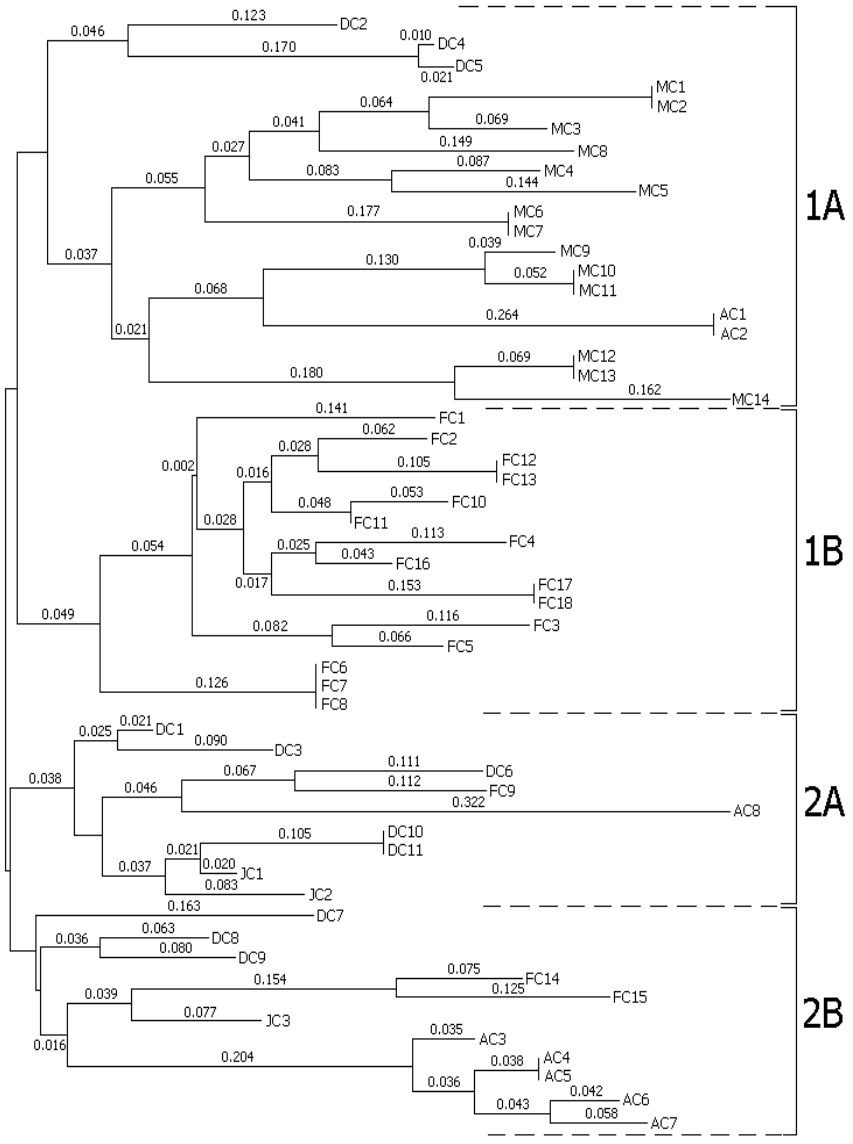

Figure 5. Dendrogram of the 54 *V. cholerae* isolates constructed using the ERIC-PCR data as presented in the neighbor joining tree (NJTree) format. Numbers represent distance value between the isolates.

BOX-PCR profiles

From the BOX-PCR profiles, a total of 39 different patterns were generated. The representative profile of the BOX-PCR obtained with BOX primer is shown in Figure 6. Based on the dendrogram (Figure 7) generated from this data, the isolates were clustered into two main clusters which consists of nine and 45 isolates, respectively. Isolates that originated from surface water sample (Sg. Bako, Sg. Tabuan and Sg. Kuap) formed a tight cluster in Cluster 1, while the other isolates were divided further into three subclusters (Clusters 2A, 2B and 2C). The average distance of Cluster 1 was found to be the most homogenous, showed a low average genetic distance, D = 0.02 whereas groups in Cluster 2 showed higher average distance, with D = 0.404. Overall, the average genetic distance among all the 54 isolates was 0.504, ranging in values from 0.00 to 0.810, indicating a high variation in the isolates tested.

Combination of RAPD-PCR, ERIC-PCR and BOX-PCR profiles

The amplification profiles obtained with the RAPD primer (GEN-01-50-09), ERIC primer (ERIC-1R and ERIC-2) and BOX primer (BOX-1R) were combined in a single dendrogram to further study the intraspecific relation among the *V. cholerae* isolates. From the dendrogram (Figure 8) generated based on the combination of the three profiles, a similarity analysis of the fingerprints yielded a grouping of isolates into three large clusters, designated as Cluster 1, 2 and 3. Cluster 1 consists of 30 isolates with average distance of D = 0.414. While all of the isolates from March, 2004 and two isolates from April, 2004 were grouped in Cluster 2 with average distance of D = 0.392. Lastly, Cluster 3 contains ten isolates, most of which were isolated from surface water sampled in April, 2004. In this cluster, the average distance of D = 0.43 was observed.

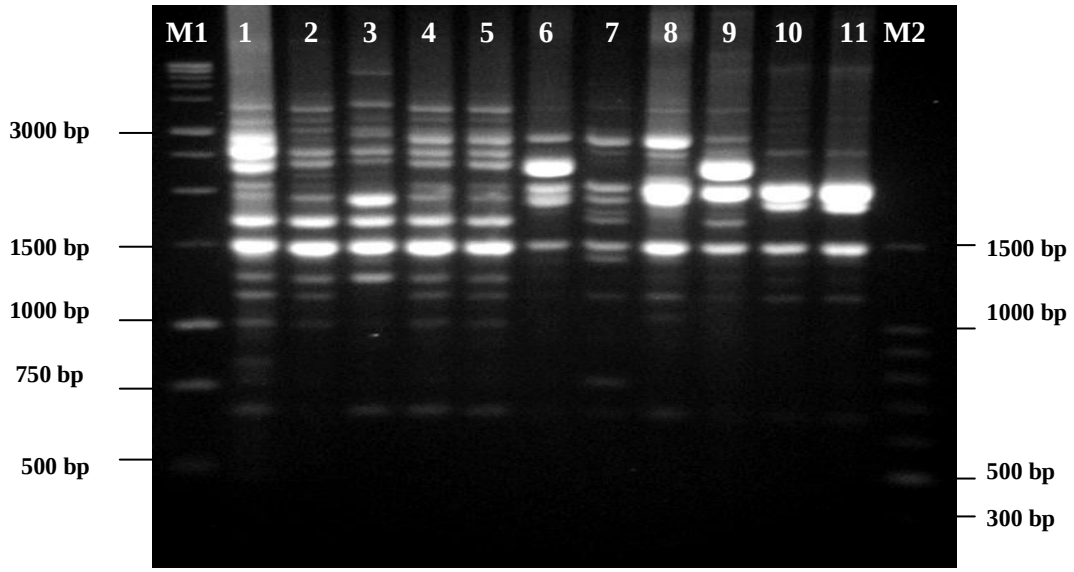

Figure 6. Agarose gel (1.2% w/v) electrophoresis of BOX-PCR fingerprints of the *V. cholerae* representative isolates obtained with primer BOXAIR. M1: 1 kb DNA ladder. Lane 1-11: DC1-DC11, M2: 100 bp DNA ladder.

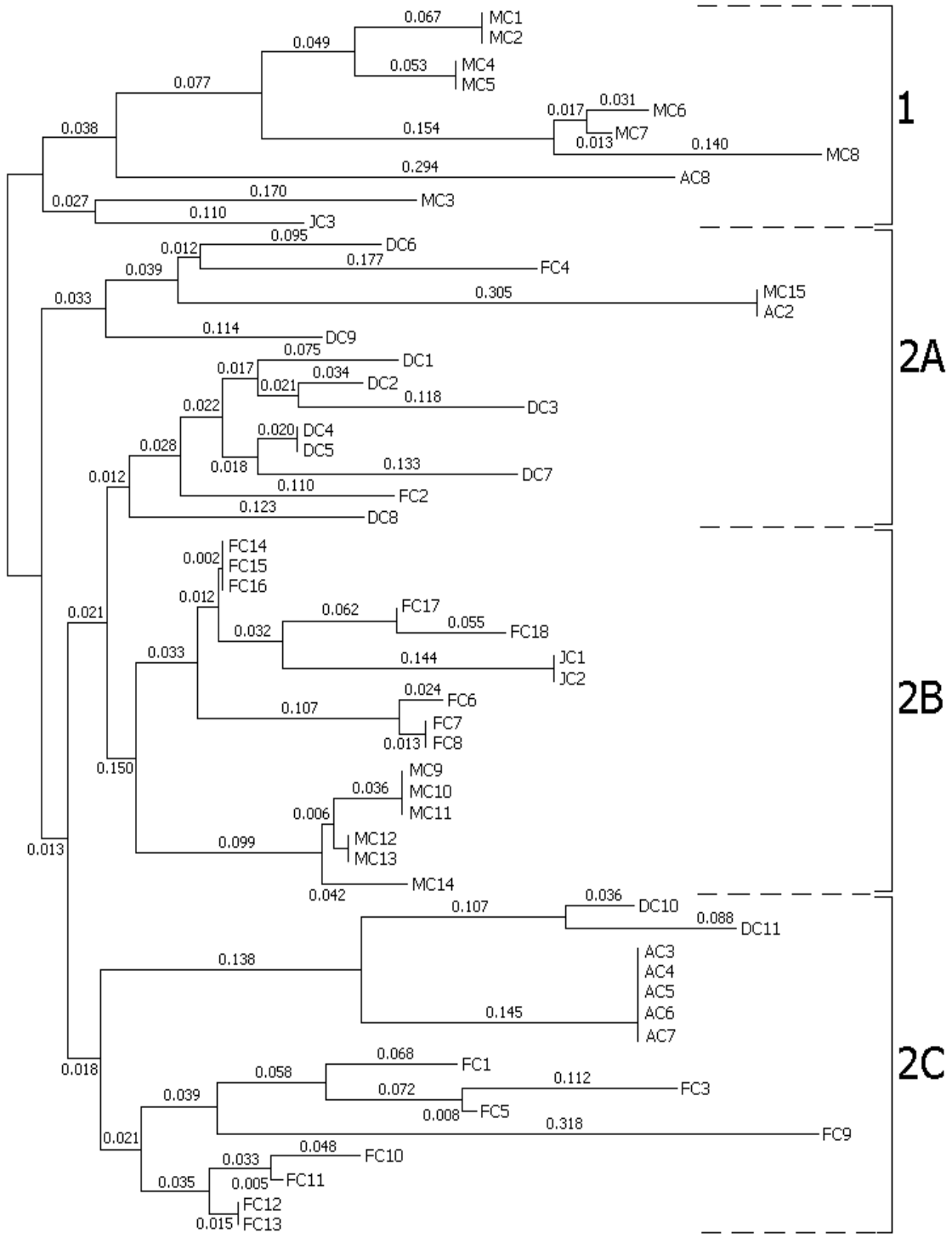

Figure 7. Dendrogram of the 54 *V. cholerae* isolates constructed using the BOX-PCR data as presented in the neighbor joining tree (NJTree) format. Numbers represent distance value between the isolates.

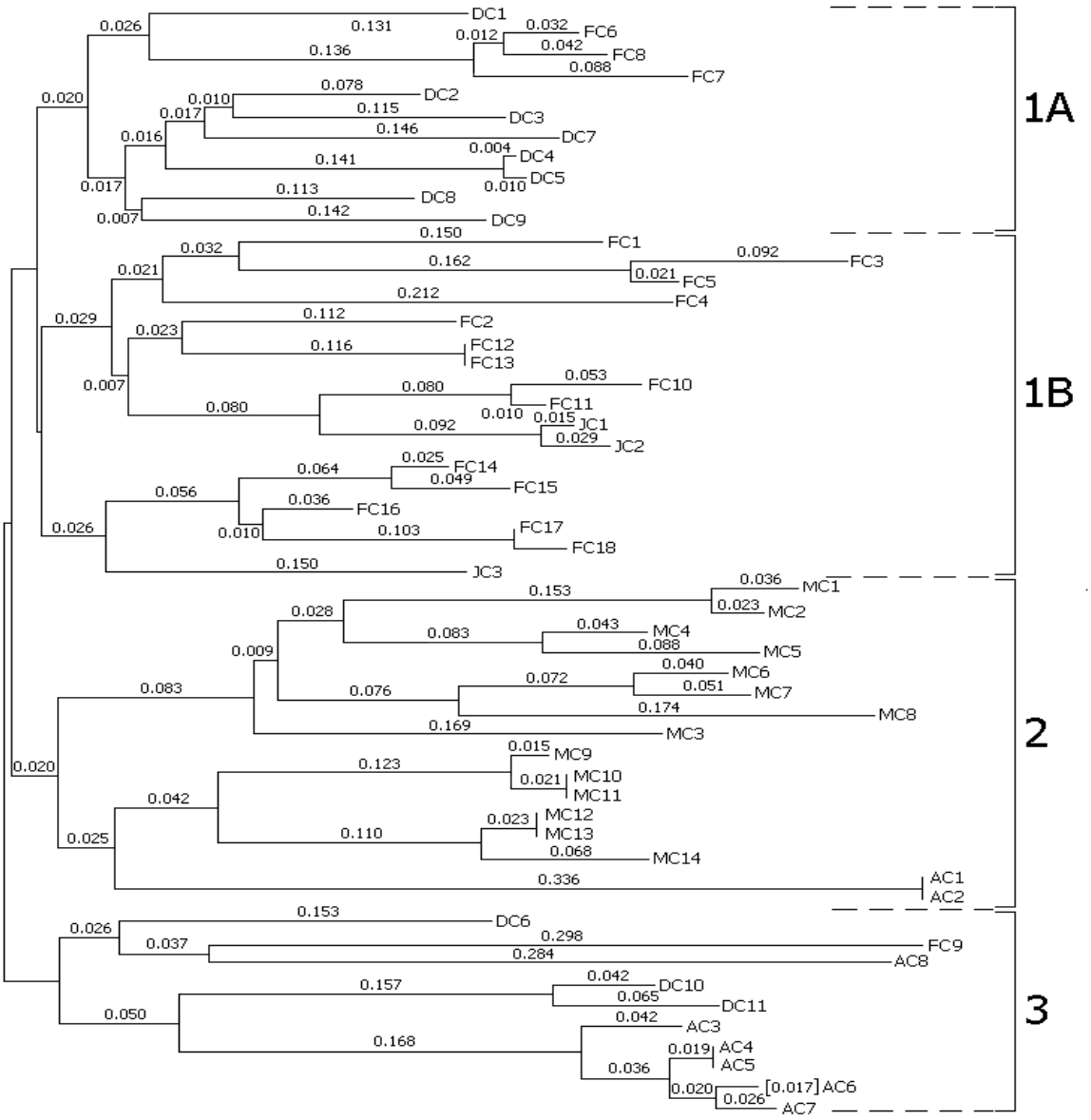

Figure 8. Dendrogram of the 54 *V. cholerae* isolates constructed using the combination of RAPD-PCR, ERIC-PCR and BOX-PCR data as presented in the neighbor joining tree (NJTree) format. Numbers represent distance value between the isolates.

Simpson's index of Diversity

In this study, Simpson's Index of Diversity was applied to measure the clonal diversity of the *V. cholerae* isolates. For each of the respective typing method, the discrimination index represents the percentage of occasions that two strains sampled randomly from a population fall into different types. The calculation of discriminatory ability is summarized in Table 3. From the results, RAPD-PCR, ERIC-PCR, BOX-PCR exhibited an excellent discrimination index of 0.982 or higher, with ERIC-PCR having the highest discriminatory index.

DISCUSSION

In this study, RAPD-PCR, ERIC-PCR and BOX-PCR were used to measure and compare the genetic relatedness of 54 *V. cholerae* non-01/non-0139 strains isolated from water sources and seafood in the Kuching-Samarahan District, Sarawak (Malaysia). The isolates were successfully differentiated into 39 types by RAPD-PCR, 43 types by ERIC-PCR, and 38 types by the BOX PCR, with the overall average polymorphic distances observed to be at 0.593, 0.527 and 0.504, respectively, based on the distance matrix values. Further analyses of the individual RAPD-PCR,

Table 3. Number of isolates with particular RAPD-PCR patterns using GEN-01-50-09.

	Simpson's Index of Diversity
RAPD-PCR	0.986
ERIC-PCR	0.992
BOX-PCR	0.987

ERIC-PCR and BOX-PCR profiles showed that only few of the isolates, even from the same sources and sampling period, shared identical genetic fingerprints. For example, only two isolates (FC12 and FC13) from Sg. Tabuan, six isolates (MC10 and MC11; AC1 and AC2; AC4 and AC5) from Tambak Sejingkat, and two isolates (MC12 and MC13) from Sg. Sejingkat showed the identical fingerprint patterns via all three assays. Whereas the remaining 44 isolates generated different fingerprint patterns, therefore exhibiting a high degree of genetic diversity among the *V. cholerae* non-01 isolates from both seafood and surface water samples.

The RAPD-PCR, ERIC-PCR and BOX-PCR profiles were used to evaluate the extent of genetic variation among the *V. cholerae* isolates via Simpson index of Diversity. This calculation is a simple mathematic measurement to observed species diversity in a community whereby values reaching 1 shows greater diversity (Hunter & Gaston, 1988). By using this formula, the index of diversity were found to be significantly high in values at 0.986 (RAPD-PCR), 0.992 (ERIC-PCR) and 0.983 (BOX-PCR) (Table 3).

Of the three PCR-based genotyping assays, ERIC-PCR showed the highest discriminatory power, an observation that is in agreement by a study done by Rivera *et al*. (1995). The same study also commented that ERIC-PCR is a method of choice when a single genotyping procedure is to be used, as not only is this assay rapid, but it was also relatively easy to perform. In addition, ERIC-PCR has also been proven to be a powerful tool for molecular analysis of other *Vibrio spp.* such as *V. alginolyticus* and *V. parahaemolyticus* (Rivera *et al.*, 1995). This assay relies on the generation of amplified products based on the frequency and orientation of the Enterobacterial Repetitive Intergenic Consensus (ERIC) sequences by using the combination of primers designed to target the conserved ERIC regions. However, the potentially rapid change in ERIC-PCR types could also obscure relationships between strains that otherwise might be evident if other methods were used (Marshall *et al.*, 1999).

In order to increase the likelihood of demonstrating strains dissimilarities, the data from the RAPD-PCR, ERIC-PCR and BOX-PCR fingerprint patterns obtained were combined into a single dendrogram (Figure 8). The dendrogram constructed from the combination of these profiles showed that the 54 isolates could be clustered into three major clusters, containing 28 (Clusters 1A and 1B), 16 (Cluster 2) and ten (Cluster 3) isolates, respectively. When the entire DNA fingerprinting results were combined, the detection of polymorphism was further enhanced and refined. This was also discussed by Al-Haddawi *et al.* (1999) that reported strains with identical banding pattern generated by a single primer set could be differentiated further with more primers or primers combinations.

An interesting observation from the combinatory dendrogram in Figure 8 shows that isolates are grouped into distinct clusters that are temporally correlated, as per sampling period. For example, isolates obtained from the months of December, 2003 to February, 2004 were clustered in the same cluster (Cluster 1). While isolates from March, 2004 are grouped in Cluster 2, and, isolates from April 2004 are grouped in Cluster 3. Only three of the December, 2003 isolates (DC6, DC10, DC11) and one from February, 2004 (FC9) are grouped separately in Cluster 3. This finding indicates that combination of the RAPD-PCR, ERIC-PCR and BOX-PCR data is able to differentiate temporal diversity.

Based on Dice formulation (Nei & Li, 1979), the genetic distances between the 54 isolates ranged from 0.00 to 0.84. The isolates FC9 and MC8 are found to be genetically most different to each another with the genetic distances of 0.84. While ten isolates (FC12 and FC13, MC10 and MC11; MC12

and MC13; AC1 and AC2; AC4 and AC5) with genetic distances equal to 0.00 indicating they are genetically similar. The data, however, does not detect any significant genetic differences between the seafood and environmental *V. cholerae* isolates in terms of sampling locations and types of samples.

The inconsistencies between isolates and their origin were also discussed by Son *et al*. (2002) on the molecular characterization of *V. cholerae* non-01/non-0139 from human and environmental sources in Malaysia. Their study also revealed a wide genetic variability among *V. cholerae* isolates, with no apparent correlation with the sources of origin. According to several other studies, while environmental and clinical strains of pathogenic 01 and 0139 *V. cholerae* are known to represent specific clones that is grouped closely with one another in a tight cluster, there is significant genetic diversity among environmental and non-01, non-0139 strains of *V. cholerae* (Bakhshi *et al*., 2009; Bhowmick *et al*., 2007; Jiang *et al.,* 2000; Lipp *et al*., 2002; Rivera *et al*., 1995). The general heterogeneity observed among the *V. cholerae* isolates might be the results of multiple environment exposure and genetic drift in their geographical areas as suggested by previous studies on the polymorphisms of *Vibrio* spp. (Son *et al*., 2002; Tapchaisri *et al*., 2007). Thus, riverine environment is expected to contain a very diverse population of *V. cholerae* strains and that only certain subsets of isolates are associated with human disease.

The application of these techniques clearly demonstrated significant genetic diversity among the *V. cholerae* non-01/non 0139 in the Kuching-Samarahan District of Sarawak (Malaysia). From the analyses of the data, we observed a variety of fingerprint patterns among the *V. cholerae* isolates, even among isolates from the same samples. In average, 90.7% of the isolates had shared no DNA fingerprinting pattern, irrespective of the location of sampling, indicating the presence of different genetic profiles of *V. cholerae* in the sample pool. The high *V. cholerae* diversity in the seafood and surface water samples suggests that different strains are circulating in the water environment and residing in the seafood sources as oppose to widespread distribution of a single strain.

We were able to rapidly perform these molecular fingerprinting assays due to the high discriminating capacities of the three procedures. Among the three PCR based methods, ERIC-PCR provided the confirmed highest discriminatory power. Our results that PCR based method provided great discriminating power, great simplicity and excellent reproducibility in typing of *V. cholerae* non-01 that may be useful for specific epidemiological studies such as investigating the source of outbreaks, the relatedness of isolates and identification of multiple isolates from either the same or different sources.

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