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Molecular typing of *Aeromonas* species using RAPD and ERIC-PCR fingerprinting

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Abstract: Two molecular typing methods were used in this study to examine the intra/inter-specific genetic relatedness among the *A. hydrophila, A. caviae* and *A. veronii biovar sobria* strains. In the analysis by RAPD-PCR and ERIC-PCR, the size for RAPD and ERIC fragments ranged from 0.25 to 10.0 kb with an average number of sixteen and eight bands, respectively. For the inter-species similarity, RAPD could generate 16 major clusters and 36 single isolates at the similarity of 30%. 10 clusters are significant since it grouped the strains according to their own species, however, ERIC-PCR could discriminate the strains into 4 significant clusters and 64 single isolates at the similarity of 50%. Moreover, there was no genetic similarity between food and environmental strains of *Aeromonas* sp. isolated from different geographical areas as well as from the same geographical area. Eighty five genotypes among the 85 *A. hydrophila, A. caviae* and *A. veronii biovar sobria* isolates were generated using RAPD and ERIC-PCR which indicated that the strains were very diverse.

Keywords: RAPD, ERIC, *A. hydrophila, A. caviae, A. veronii* biovar sobria

Introduction

The genus *Aeromonas* comprises several species of oxidase negative and catalase positive, glucose-fermenting, facultative anaerobic, gram-negative, rod-shaped, motile and non-motile bacteria. They are widely distributed in nature, especially in the aquatic environment and have been isolated from a variety of raw food. According to Guadalupe *et al*. (2005), several species of the genera have been associated with several diseases in both warm- and cold-blood animals. In humans, they are opportunistic pathogens causing gastroenteritis, and less commonly, cellulitis, wound infections, meningitis, otitis, peritonitis, endocarditis and septicemia (Janda *et al*., 2001).

Studies involving both the clinical and environmental *Aeromonas* strains using ribotyping have indicated that apparently unrelated strains can show identical laborious nature may be a shortcoming of the technique. Pulsed-field gel electrophoresis (PFGE) has been considered to be one of the best typing methods (Alavandi *et al*., 2001). However, when compared with the RAPDs for typing *A. hydrophila* isolates, the latter was simpler, cheaper, and quicker to perform, and consequently more suitable for the epidemiological studies (Talon *et al*., 1998). Recently, the restriction fragment length polymorphism (RFLP) of the 16S-23S intergenic spacer region (ISR) has been used successfully for typing the *Aeromonas veronii* strains (Mart´ınez-Murcia *et al*., 2000). However, this technique is laborious as compared to the other typing methods based on the PCR amplification of short and repetitive sequences present on the genome of bacteria, such as enterobacterial repetitive intergenic consensus (ERIC) and repetitive extragenic palindromic units (REP) (Versalovic *et al*., 1991). These motifs are genetically stable and differ only in their copy number and chromosomal locations, making them a good target for strain differentiation (Hulton *et al*., 1991). Both RAPD and ERIC were used for typing *Aeromonas* isolates and both methods proved to be effective for demonstrating a nosocomial

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transmission of *A. hydrophila* (Soler *et al*., 2003). In spite of that, there are only a few comparative studies on the reproducibility and discriminatory power of the molecular typing methods in *Aeromonas*.

Materials and Methods

Bacterial strains

Thirty eighty isolates of *Aeromonas hydrophila*, *A. caviae* (38 isolates) and *A. veronii* biovar *sobria* (9 isolates) isolated from the fresh water and seafood in the different locations in Malaysia were examined for the RAPD-PCR and ERIC-PCR analysis to determine their relatedness.

Genomic DNA isolation

A modification of the method of Murry and Thompson (1980) was used for the DNA extraction from the *Aeromonas* strains. An overnight culture in a LB broth was centrifuged in a 1.5 ml microcentrifuge to harvest the cell pellet. The cell pellet was then resuspended in the 700 µl GET buffer. Later, 5 µl Proteinase K (25 mg/ml) and 10 µl 25% SDS (sodium dodecyl sulphate) were added to the cell solution and mixed gently. The tube was incubated at 60ºC for 20 minutes to lyse the cell. After incubation, 500 µl PCI (phenol-chloroform-isoamyl) solutions was added and centrifuged at 12000 rpm for 1 minute. 200 µl of the clear upper layer solution was transferred into a new tube before adding 200 µl KAc and 400 µl cold isopropanol. The mixture solution was then put on ice for 5-10 minutes. The precipitated DNA was recovered by centrifugation at 12000 rpm for 10 minutes. The supernatant was discarded and the pellet was washed with 500 µl of cold 70% ethanol. The pellet was air-dried and dissolved in 30 µl of sterile distilled water before using it as the DNA template for the RAPD-PCR and ERIC-PCR.

RAPD-PCR amplification

Ten random primers having the 50% G+C contents gene sequence (10-mer) were screened and three primers, the Gen 1-50-01 (5'-GTG CAA TGA G-3'), Gen 1-50-02 (5'-CAA TGC GTC T-3') and Gen 1-50-09 (5'-AGA AGC GAT G-3') were selected for further study as they provided reproducible and discriminatory pattern. The amplification reactions were performed in 25 ml volume containing 2.5 mM $MgCl_2$, 200 mM each dATP, dCTP, dGTP and dTTP (Promega), 0.5 mM primer, 1.25 Units of *Taq* polymerase, 10-20 ng of genomic DNA. These amplifications were carried out in the thermal cycler (Perkin Elmer Cetus 2400), the cycling program was 94ºC for 1 minute, 36ºC for 1 minute and 72ºC for 2 minutes, continuing for 45 cycles. A final elongation step at 72ºC for 5 minutes was included. The amplification products were fractionated by the electrophoresis through 1.5% agarose gel and detected by staining with ethidium bromide and photographed under UV light using Gel Doc 2000 (Bio Rad).

ERIC – PCR amplification

The fingerprint patterns of the strains were determined by using both the oligonucleotide ERIC-1R (5'-ATGTAAGCTCCTGGGGATTCAC-3') and ERIC-2 (5'-AAGTAAGTGACTGGGGTGAGCG-3') primers, as described by Versalovic *et al.* (1991). These primers were obtained from Genosys Biotechnologies Inc. The PCR technique was carried out in 0.2 ml microcentrifuge tubes, with 25 µl of reaction mixture consisting of 7.5 µl sterile distilled water, 2.5 µl 10x PCR buffer, 1.5 µl 25 mM $MgCl_2$, 0.5 µl 2.5 mM deoxyribonucleotide phosphate (dNTP), 100 pmol of each of the ERIC-1-R and ERIC-2 primers (1.0 ml each) and 2.5 U of *Taq* DNA polymerase (1.0 µl) and template DNA 10 µl. The solution mixtures were placed in a thermocycler (Perkin Elmer 2400 DNA thermal cycler). The cycling conditions were as follows: denaturation at 92°C for 45 seconds, annealing at 52°C for 1 minute and extension at 70°C for 10 minutes, with a final extension at 70°C for 20 minutes at the end of 35 cycles. For the detection and confirmation of the PCR products by gel electrophoresis, 10 µl of the amplification product mixture was subjected to electrophoresis through 1.5% agarose gel. The amplified DNA fragments of specific sizes were visualized by UV light using Gel Doc 2000 (Bio Rad) after being stained with ethidium bromide.

Results

The combination of the primers, GEN 1-50-01, primer GEN 1-50-02 and primer GEN 1-50-09 was used to show the intra species similarity of the isolates. The results in the form of a dendrogram was constructed using the RAPD Distance Software (version 1.04). *A. hydrophila, A. caviae* and *A. veronii* biovar *sobria* isolates can be divided into three, four and three major clusters, respectively. The combination of the primers, GEN 1-50-01, primer GEN 1-50-02 and primer GEN 1-50-09 and *A. hydrophila, A. caviae* and *A. veronii* biovar *sobria* isolates was used to show the inter species similarity of the isolates which generated three major clusters.

For the ERIC-PCR analysis, the results were analyzed with the RAPD Distance software. As for the intra species diversity *A. hydrophila, A. caviae* and *A. veronii* biovar *sobria* isolates can be divided into three, four and three major clusters, respectively. The combination of the *A. hydrophila, A. caviae* and *A. veronii* biovar *sobria* ERIC results could discriminate them into three major clusters.

Discussion

Studies on the origin and fingerprint of the *Aeromonas* species have become very important because of the increasing incidence of infections caused by this organism worldwide. The RAPD-PCR test is very powerful and more sensitive than other typing techniques (Welsh and McClelland, 1990). Currently, molecular techniques have been used as the typing procedures for the epidemiological studies of both clinical and environmental *Aeromonas* strains (O'hlic *et al*., 2000; Szczuka and Kaznowski, 2004). In this work, the RAPD and ERIC confirmed their discriminatory power to distinguish clones amongst the analyzed isolates. Although there are some arguments against the reproducibility of the PCR-based typing methods (Van Belkum, 1997) in the present study, these methodologies were highly reproducible in the overall banding patterns and band repetition, concurring with other studies in our laboratory (Sahilah *et al*., 2008; Lee *at al*., 2008; Zulkifli *et al*., 2009; Tan *et al*., 2009).

The results in this study showed that the RAPD provided a high diversity of polymorphism between *A. hydrophila* strains isolated from the different sources and locations in Malaysia, with the PCR products ranging below 0.25 to 10.0 kb. For certain isolates, they failed to produce any products with primers used and these were referred as untypeable. A few discrepancies in the classification results obtained by each primer may be explained by the fact that the 10-mer primers detected polymorphisms occurring in distinct chromosomal regions and, therefore, variations in one region may not necessarily affected other regions (Nociari *et al*., 1996). One possible explanation could be the amplification of the DNA loci displaying a high frequency of mutation or rearrangements caused by intra- or inter-strain exchange of the genetic material (Sinha *et al*., 2004). Therefore, the combination of data obtained by the different primers or typing procedures gave the optimal insight into strain relatedness (Sinha *et al*., 2004).

The result was analyzed using the RAPD-Distance software to construct the phylogenetic dendrogram and the relationship between the *A. hydrophila* strains could be estimated. The data from the three primers were used to generate a distance matrix and from the dendrogram generated, all the strains were divided into 3 major clusters. Cluster A – contained 5 strains which had been isolated from the same source (shrimp), Cluster B – 6 strains which were isolated from the same location (UPM ponds), Cluster C – 27 strains but these strains were isolated from the different sources and locations, with the similarity between 0% to 52% (data not shown).

The dendrograms of 38 and 9 isolates of the *A. caviae* and *A. veronii* biovar *sobria,* respectively obtained from the combination of the primer GEN 1-50-01, primer GEN 1-50-02 and primer GEN 1-50-09. All the *A. cavia* strains can be divided into 2 significant clusters, Cluster A-2 strains which were isolated from the same source (fresh water samples obtained from the UPM ponds) , Cluster B-2 strains, which were isolated from the same location (tiger prawn farm) and 34 single isolates at the similarity of 62%. For *A. sobria,* the combination of the primers could discriminate the strains into one significant cluster which grouped the three strains isolated from the same source (shrimp samples) and six single isolates at the similarity of 40%. The results of the RAPD profiles of the *Aeromonas* isolates from the food and environmental sources strongly suggested that the *Aeromonas* isolates possess a high degree of genetic diversity within the same species.

The combination of the primers, GEN 1-50-01, primer GEN 1-50-02 and primer GEN 1-50-09 and *A. hydrophila, A. caviae* and *A. veronii* biovar *sobria* isolates were used to show the inter species similarity of the isolates which generated 16 major clusters and 36 single isolates at the similarity of 30%. 10 clusters are significant since it grouped the strains according to their own species, cluster A-2 *A. caviae* strains, cluster B-4 *A. hydrophila* strains, cluster C-2 *A. caviae* strains, cluster D-2 *A. hydrophila* strains, cluster E-6 *A. hydrophila* strains, cluster F-3 *A. hydrophila* strains, cluster G-2 *A. caviae* strains, cluster H-2 *A. caviae* strains, cluster I-2 *A. hydrophila* strains, cluster J-2 *A. veronii* biovar *sobria* strains (Figure 3). The results obtained from this study showed that some strains isolated from the same geographical area isolated from the food and environment sources displayed the same pattern profile which suggested a clonal relationship.

All *A. hydrophila*, *A. caviae* and *A. veronii* biovar *sobria* strains showed distinct pattern with the expectation of the two *A. hydrophila* strains which had 100% similarity, this result is in agreement with Soler *et al*. (2002) who used three typing methods,

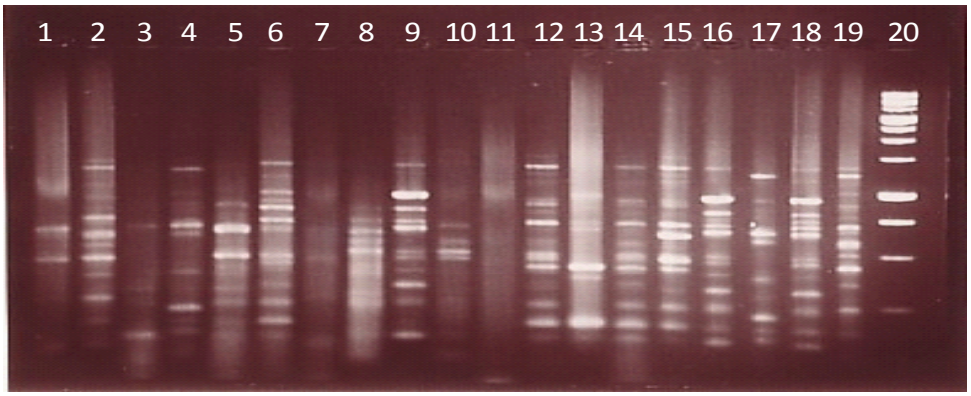

Figure 1. Representative of the RAPD fingerprints of *A. caviae* with primer Gen 1-50-09 on 1.5% agarose gel

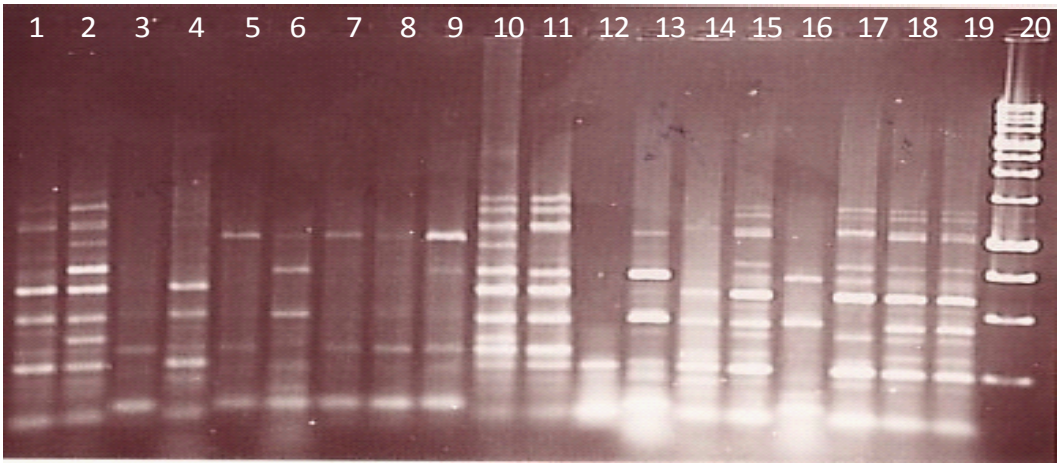

Figure 2. Representative of ERIC-PCR fingerprints of *A. hydrophila* with primer Gen 1-50-09 on 1.5% agarose gel

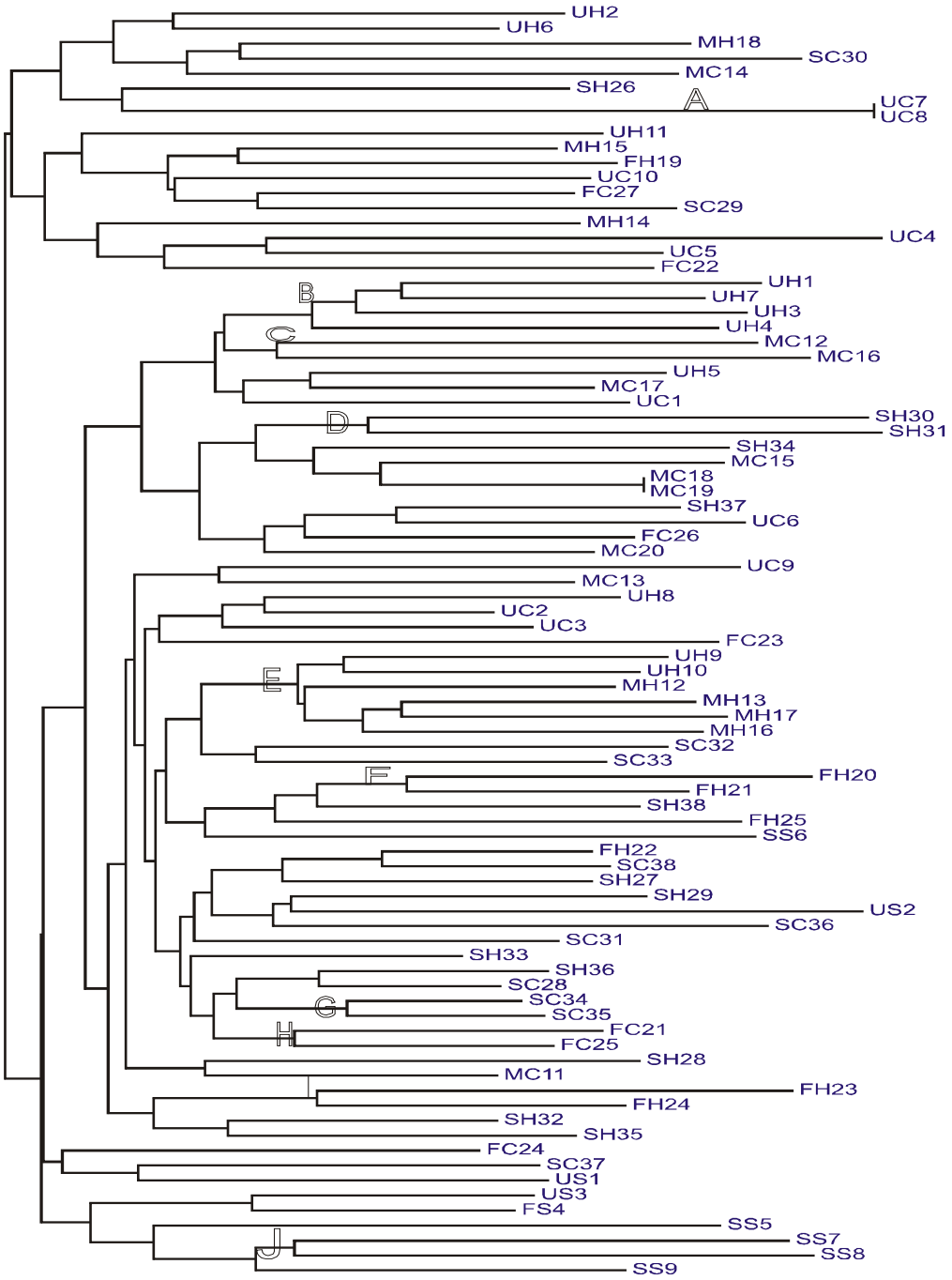

Figure 3. Dendrogram showing RAPD profiles of typeable *A. hydrophila, A. caviae* and *A. veronii* biovar *sobria* isolates using GEN1-50-01, GEN1-50-02 and GEN1-50-09

Note: SH: *A. hydrophila* isolated from shrimp, FH: *A. hydrophila* isolated from fish, MH: *A. hydrophila* isolated from Tiger Prawn Farm, UH: *A. hydrophila* isolated from UPM ponds. FC: *A. caviae* isolated from fish, UC: *A. caviae* isolated from UPM ponds, MC: *A. caviae* isolated from Tiger Prawn Farm in Malacca, SC: *A. caviae* isolated from shrimp. FS: *A. veronii* biovar sobria isolated from fish, SS: *A. veronii* biovar sobria isolated from shrimp, US: *A. veronii* biovar sobria isolated from UPM ponds

restriction fragment length polymorphism (RFLP), PCR amplification of the enterbacterial repetitive intergenic consensus (ERIC) and the repetitive extragenic palindromic unite (RFP) for typing 26 isolates of *A. popoffii* from the different geographical origins, with the ERIC showed the highest discriminatory power.

For the inter-species similarity, the ERIC-PCR could discriminate the strains into 4 significant clusters and 64 single isolates at the similarity of 50%., cluster A-4 *A. hydrophila* strains, clustermB-2 *A. caviae* strains, cluster C-4 *A. hydrophila* strains, cluster D-3 *A. caviae* strains (Figure 4). Some authors indicated that water contaminated with *Aeromonas* spp. could be a source of fish disease (Altwegg *et al*., 1999). However, our results revealed that the clones of *Aeromonas* sp. isolated from the fish in UPM ponds did not show a close genetic similarity with the clones isolated from the water samples retrieved from the ponds. Although there are indications of a high diversity among the strains population, we could find some strains which isolated from food and from environment were equal or highly similar. This finding is relevant because it shows that they share the same genotype.

Previous reports have shown that RAPD profiles

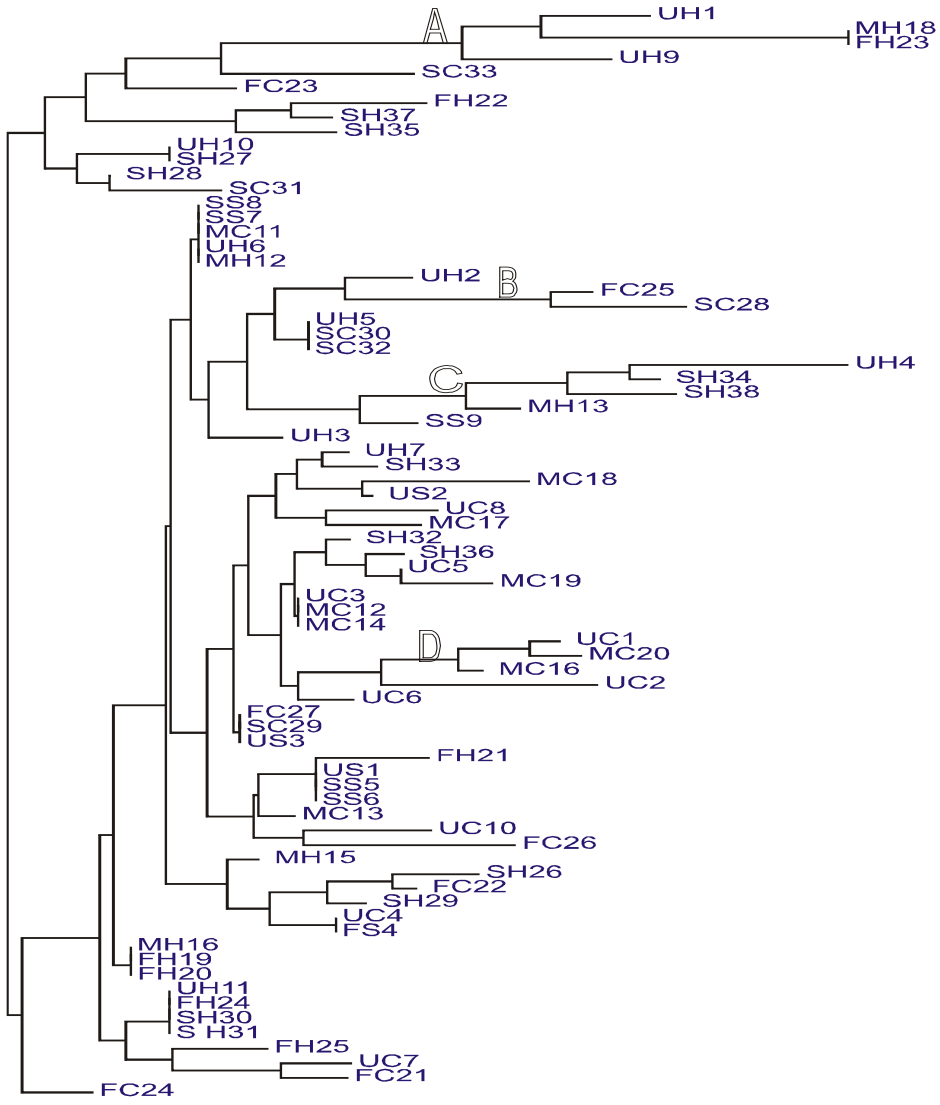

Figure 4. Dendrogram showing ERIC profiles of typeable *A. hydrophila* , *A. caviae* and *A. veronii* biovar *sobria* isolates using ERIC primer

Note: SH: *A. hydrophila* isolated from shrimp, FH: *A. hydrophila* isolated from fish, MH: *A. hydrophila* isolated from Tiger Prawn Farm, UH: *A. hydrophila* isolated from UPM ponds. FC: *A. caviae* isolated from fish, UC: *A. caviae* isolated from UPM ponds, MC: *A. caviae* isolated from Tiger Prawn Farm in Malacca, SC: *A. caviae* isolated from shrimp. FS: *A. veronii* biovar sobria isolated from fish, SS: *A. veronii* biovar sobria isolated from shrimp , US: *A. veronii* biovar sobria isolated from UPM ponds

generated from the *A. salmonicida* subsp. *salmonicida* strains revealed a genetic homogeneity amongst the strains from several locations, whereas the *A. hydrophila* strains showed a relatively high diversity, as inferred from the number of observed genotypes (Miyata *et al*., 1995). The intra-specific diversity of *A. hydrophila* strains generated by both the RAPD and ERIC obtained in this work was high, indicating a considerable diversity within this species.

Thus, the RAPD and ERIC profiles should not be used to differentiate species; rather their use as the typing tools should be supported (O'hlic *et al*., 2000; Solar *et al*., 2003; Szczuka and Kaznowski, 2004). The present data obtained in this study are in total agreement with the previous findings (Miyata *et al*., 1995; Sechi *et al*., 2002; Soler *et al*., 2003; Guadalupe *et al*., 2005).

The rationale for performing the molecular typing was to understand whether any particular clone of the *Aeromonas* species was more often among all the *A. hydrophila, A. caviae* and *A. veronii* biovar *sobria* isolated from the food and environment. Except for a few strains, both the RAPD-PCR and ERIC-PCR showed that the *Aeromonas* strains were genetically heterogeneous and that no particular clone was predominant. In summary, the RAPD-PCR and ERIC PCR methods can be used to fingerprint the *Aeromonas* species isolates from the food and environmental samples and are much easier to use as compared to other typing methods. The molecular typing method described in this study could be used to aid trace-back investigations aimed at determining the source of improperly handled food as well as to create opportunities for intervention or remediation

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