UNIVERSITI PUTRA MALAYSIA

CHARACTERIZATION OF 16S rRNA AND INTERNAL TRANSCRIBED SPACER (ITS) REGION GENE SEQUENCING OF THE AEROMONAS SPECIES ISOLATED FROM CULTURED FRESHWATER FISHES

DIYANA NADHIRAH BINTI KHAIRUL PARMAN

FP 2013 87

CHARACTERIZATION OF 16S rRNA AND INTERNAL TRANSCRIBED SPACER (ITS) REGION GENE SEQUENCING OF THE *AEROMONAS* SPECIES ISOLATED FROM CULTURED FRESHWATER FISHES

DIYANA NADHIRAH BINTI KHAIRUL PARMAN
157226

This project report is submitted in partial fulfillment of the requirement for degree of Bachelor of Agriculture (Aquaculture)

DEPARTMENT OF AQUACULTURE
FACULTY OF AQUACULTURE
UNIVERSITY PUTRA MALAYSIA
SERDANG, SELANGOR

2013

CERTIFICATION OF APPROVAL

DEPARTMENT OF AQUACULTURE

FACULTY OF AGRICULTURE

UNIVERSITI PUTRA MALAYSIA

Name of student : Diyana Nadhirah Binti Khairul Parman.

Matric number : 157226

Programme : Bachelor of Agriculture (Aquaculture)

Year : 2013

Name of supervisor : Dr. Ina Salwany Binti Md. Yasin

Title of Project : Characterization of 16S rRNA and Internal Transcribed Spacer (ITS) Region Gene Sequencing of the *Aeromonas* Species Isolated From Cultured Freshwater Fishes

This is to certify that I have examined the final project report and all corrections have been made as recommended by the panel of examiners. This report complies with the recommended format stipulated in the AKU4999 project guidelines, Department of Aquaculture, Faculty of Agriculture, University Putra Malaysia.

Signature and official stamp of supervisor/& cosupervisor:

_____

Supervisor's name
Date:

ACKNOWLEDGEMENTS

First of all, thanks to ALLAH S.W.T for his mercy and guidance in giving me full strength to finished my "Final Year Project". There are lots of difficulties and also hard works given to complete this project. Alhamdulillah this project has been done in success and on time.

I would like to express my greatest gratitude to my final year project supervisor, Dr. Ina Salwany Md Yasin for her constant supervision, advice, guidance and encouragement from the beginning until the end of my project.

The most I am grateful to my laboratory mate during finished my project are Miss Zarirah Zulperi and Miss Saleema Matusin both of them were helping me a lot in teaching me and helping me the most of time. Moreover, thanks to the Malaysian National Fish Health Research Centre (Nafish) staffs. My gratitude goes to my course mates and friends who had sharing their thought, ideas, and supports during completing this project. I am also grateful to my friends, Rabi'atul 'Adawiyah Sezali, Muhammad Pippudin Abdul Aziz and Nehlah Rosli for their kindness in helping me in culturing the bacteria samples. Last but not least, I am grateful to all the staffs of Aquaculture Department.

Lastly, greatest gratitude and thankful to my parents for giving me moral support and help me in term of financial while doing my project.

ABSTRACT

The *Aeromonas* species are significantly found mostly on freshwater fishes that cause of disease outbreak that could not be easily control and determine their species in a rapid time. Twenty-two of *Aeromonas* strains isolated from diseased freshwater fishes were identified by using the 16S rRNA and Internal Transcribed Spacer (ITS) region gene sequencing. Thus all these strains were sequenced then performed a phylogenetic analysis by comparing the sequences which obtain from the BLASTn program by using methods of neighbor-joining and bootstrap value to compute the powerful topology. According to this study, the gene sequences ranged fragment size for 16S rRNA was 1500 bp and the Internal Transcribed Spacer (ITS) region was between 1000 bp to 1200 bp. All these 22 strains were identified up to genus level by 16S rRNA gene as *A. hydrophila* (15), *A. veronii* (7). However, ITS gene sequencing showed identification up to the species level as the *A. hydrophila* (14), *A. veronii* (8). Therefore, this PCR method has found to be simple, easy to perform and faster in identified the *Aeromonas* species. Moreover it can be used efficiently for regular monitoring of *Aeromonas* species when an outbreak happens.

ABSTRAK

Spesis *Aeromonas* kebanyakannya sering didapati pada ikan air tawar yang menyebabkan penyakit yang tidak boleh dikawal dengan mudah dan tidak mudah untuk menentukan spesis dalam masa yang singkat. Dua puluh dua strain *Aeromonas* diasingkan daripada ikan air tawar yang berpenyakit telah dikenal pasti dengan menggunakan 16S rRNA dan "Intergenic Transcribed Spacer" (ITS) gen jujukan. Oleh itu, kesemua strain ini telah dijujukkan dan kemudiannya diteruskan kepada analisis filogenetik dengan membandingkan jujukan yang di dapati dari BLASTn dengan menggunakan kaedah "Neighbor-joining" dan nilai Bootstrap untuk menghasilkan topologi yang terbaik. Menurut kajian ini, julat serpihan saiz jujukan gen bagi 16S rRNA adalah 1500 bp dan "Internal Transcribed Spacer" (ITS) adalah di antara 1000 sehingga 1200 bp. Kesemua 22 strain in telah dikenal pasti sehingga ke peringkat genus bagi 16S rRNA gen adalah *A. hydrophila* (15), *A. veronii* (7). Kesemua strain ini yang telah menggunakan gen ITS menunjukkan pengesahan sehingga ke peringkat spesis sebagai *A. hydrophila* (14), *A. veronii* (8). Oleh itu, kaedah PCR ini telah didapati lebih mudah dan cepat untuk mengenalpasti spesis *Aeromonas* . Selain itu ianya boleh digunakan dengan berkesan untuk pemantauan berkala bagi spesis *Aeromonas* sekiranya wabak berlaku.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

GDP	Gross domestic product
US	United state
USA	United States of America
MA	Massachusetts
Mich	Michigan
NJ	New jersey
NaFish	National Fish Health Research Centre
DNA	Deoxyribonucleic Acid
RNA	Ribonucleic Acid
rRNA	Ribosomal Ribonucleic Acid
UV	Ultraviolet
bp	Base pair
kb	Kilobase
mM	Milimolar
mL	Milliliter
ng	Nanogram
pmol	Picomoles
rpm	Revolution per minute
µL	Microliter
µM	Micromolar
^{o}C	Degree celcius
%	Positive
+ve	Percentage
-ve	Negative

CHAPTER 1

INTRODUCTION

Aquaculture can be defined as the nurture of aquatic organisms such as fish, mollusks, crustaceans, aquatic plants, crocodiles, alligators, turtles and amphibians. Farming implicit some types of intervention in the rearing process to enhance production, such as feeding and protection from predators. It is also involving the individual or corporate ownership of the stock being cultured (FAO, 2001).

Austin and Austin (2012) has stated that the fish diseases was in intense situations which causes to problems in fish farms where there are outbreaks either begin suddenly, rapidly with the high of mortality and subside with equal promptness that is known as an acute disease or spread slowly with less harshness but keep on longer time known as chronic disease. Nowadays, most of aquaculture production has significantly increasing in many Asian countries, since then it also came along with the environmental and socioeconomic impacts which the most important are fish health issues. As well been familiar to the aquaculture today is a fish disease problems that cause to the factor of millions of dollars lost annually in commercial aquaculture. Therefore, it is important to understanding the epidemiology, etiology and ecology of the infectious agents that causes the mass mortalities in aquaculture production, especially in *Aeromonas* species that mostly caused to the chronic diseases to the Tilapia species and other species too (Nielsen *et al.*, 2001). There are consists of two genus of *Aeromonas* which is non-motile species as an

example like *Aeromonas salmonicida* and motile species like *Aeromonas hydrophila*.

According to Siti-Zahrah et al. (2005), *Aeromonas* species have causes the disease outbreaks cases reported in 1997 at Temerloh, Pahang, where the cage culture of fish in Sungai Pahang had increase in susceptibility. This case study revealed there was highly mortality rate that cause by heat-stress related syndrome and the presence of *Aeromonas* species, *Aeromonas hydrophila* from kidney of fish. There are reported in June, 2000 where there was highly mortality or outbreak in tilapia species which is reported every year during a dry season. Refers to Bergey (1994), as in the identification and characterization of the *Aeromonas* species came so important towards aquaculture industries today, these species identified by using the biochemical analysis and change to molecular analysis due to biochemical identification may need to resort to relatively difficult procedures to achieve an accurate identification. Refer to Parker and Shaw (2011) as to overcome this lack, the molecular techniques have been developed to identify these species but there is limitation using it which is many of the DNA probes for *Aeromonas* have a very narrow spectrum allowing only one species to be identified at a time. Therefore, this study is established to fulfill the following objective:

1. To genetically identify and compare similarity of *Aeromonas* species isolated from diseased of freshwater fishes by using Internal Transcribed Spacer (ITS) region and 16S rRNA gene sequencing.

REFERENCES

Abbott S.L., Cheung W.K. and Janda J.M. (2003). The genus *Aeromonas*: biochemical characteristics, atypical reactions, and phenotypic identification schemes. *J. Clin. Microbiol.* **41**: 2348- 2357.

Amann, R.I., Lin, C., Key, R., Montgomery, L. and Stahl, D.A. (1992). Diversity among brobacter isolates: towards a phylogenetic classification. *Syst. Appl. Microbiol*. **15**: 23-31.

Austin, B. and Austin, D. D. A. (2012). Bacterial fish pathogens: diseases of farmed and wild fish. *Springer*.

Ash, C., Martinez-Murcia, A.J. and Coliins, M.D. (1993). Identification of *Aeromonas schubertii* and *Aeromonas jandei* by using polymerase chain reaction-probe test. *FEMS Microbiol. Lett*. **2**: 80-86.

Beaz-Hidalgo, R., Alperi, A., Bujan, N., Romalde, J.L. and Figueras, M.J. (2010). Comparison of phenotypical and genetic identification of *Aeromonas* strains isolated from diseased fish. *Syst. Appl. Microbiol*. **33**: 149-153.

Bergey, D.H., (1994). Shorter Bergey's Manual of Determinative Bacteriology. *Lippincott Williams and Wilkins*. Maryland.

Berridge, B.R., Bercovier, H. and Frelier, P.F. (2001). *Streptococcus agalactiae* and *Streptococcus difficile* 16S–23S intergenic rDNA: genetic homogeneity and species-specific PCR. *Vet Microbiol.* **78**: 165-173.

Bondad-Reantaso, M. G., Subasinghe, R. P., Arthur, J. R., Ogawa, K., Chinabut, S., Adlard, R. and Shariff, M. (2005). Disease and health management in Asian aquaculture. *Vet. parasitol*. ***132***(3): 249-272.

Borrell, N., Acinas, S.G., Figueras, M.J. and Martinez-Murcia, A.J., (1997). Identification of *Aeromonas* clinical isolates by restriction fragment length polymorphism of PCR-amplified 16S rRNA gene. *J. Clin. Microbiol*. **35**: 167 – 1674.

Castro-Escarpulli, G., Figueras, M.J., Aguilera-Arreola, G., Soler, L., Fernandez-Rendon, E., Aparicio, G.O. and Chacon, M.R. (2003). Characterisation of *Aeromonas* spp. isolated from frozen fish intended for human consumption in Mexico. *Int. J. Food Microbiol.* **84**(1): 41-49.

Clarridge, J.E. (2004). Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. *Clin. Microbiol. Rev.* **17**(4): 840-862.

Corry, J.E.L., Curtis, G.D.W. and Baird, R.M. (2003). Handbook of Culture Media for Food Microbiology: Volume **34**: Progress in Ind. Microbiology. Amsterdam, the Netherlands: *Elsevier Science BV*.

Department of Fisheries. (2004). Annual fisheries statistics 2004a., Ministry of Agriculture and Agro-based Industry Malaysia. http://www.dof.gov.my/en/fishery-statistics. Retrieved April 8, 2013.

Department of Fisheries. (2012). Annual fisheries statistics 2012. Ministry of Agriculture and Agro-based Industry Malaysia. http://www.dof.gov.my/en/fishery-statistics . Retrieved January 1, 2014.

Dorsch, M., Ashbolt, N.J., Cox, P.T. and Goodman, A.E. (1994). Rapid identification of *Aeromonas* species using 16S rDNA targeted oligonucleotide primers: a molecular approach based on screening of environmental osilates. *J. Appl. Bacteriol*. **77**: 722-726.

FAO. (2001). "Aquaculture production, 2001" FAO Yearbook of Fishery Stastics. Rome, FAO, 2003. **92**(2): 186.

FAO. (2001-2013). Food and Agricultural Organization Aquaculture topics and activities. Foods and Agricultural Organization of the Nation, Rome, Italy. http://www.fao.org/fishery/legalframework/nalo_malaysia/en. Retrieved April 9, 2013.

Figueras, M.J., Soler, L., Chacon, M. R., Guarro, J. and Martinez-Murcia, A.J. (2000). Extended method for discrimination of *Aeromonas* spp. by 16S rDNA RFLP analysis. *Int. J. Syst. Evol. Microbiol.* **50**(6): 2069-2073.

Francis-Floyd, R. (2002). *Aeromonas* Infections. *Inst. Food Agric. Sci*. University of Florida, USA.

Francis-Floyd, R. (2005). Introduction to Fish Health Management, CIR 921: *Institute of INT'L. J. Agr. Rural Dev. Food Agr. Sci.* University of Florida, USA.

Garcia-Martinez, J., Acinas, S.G., Anton, A.I. and Rodriguez-Valera, F. (1999). Use of the 16S–23S ribosomal genes spacer region in studies of prokaryotic diversity. *J. Microbiol. Methods*. **36**(1): 55-64.

Gonzalez, S.F., Krug, M.J., Nielsen, M.E., Santos, Y. and Call, D.R. (2004). Simultaneous detection of marine fish pathogens by using multiplex PCR and a DNA microarray. *J. Clin.Microbiol.* **42**(4): 1414-1419.

Goodfellow, M. and O'Donnell, A.G. (1993). Roots of bacterial systematics. In: Handbook of New Bacterial Systematics (Goodfellow,M. and O'Donnell, A.G., Eds.). *Academic Press Ltd., London*. 3-54

Gurtler, V. and Stanisich, V.A. (1996). New approaches to typing and identification of bacteria using the 16S–23S rDNA spacer region. *Microbiol*. **142**: 1255–1265

Hassan, A.A., Khan, I.U., Abdulmawjood, A. and Lammler, C. (2001). Evaluation of PCR methods for rapid identification and differentiation of *Streptococcus uberis* and *Streptococcus parauberis. J Clin Microbiol* **39**: 1618–1621.

Janda, J.M., and Abbott, S.L. (2010). The genus *Aeromonas*: taxonomy, pathogenicity, and infection. *J. Clin. Microbiol. Rev*. **23**(1): 35-73.

Jensen, M.A., Webster, J.A. and Straus, N. (1993). Rapid identification of bacteria on the basis of polymerase chain reaction-amplified ribosomal DNA spacer polymorphisms. *J. Appl. Environ. Microb.* **59**: 945–952.

Kong, R.Y.C., Pelling, A. and So, C.L. (1999). Identification of oligonucleotide primers targeted at the 16S–23S rDNA intergenic spacers for genus-and species-specific detection of *Aeromonads*. *Marine pollution bulletin*. **38**(9): 802-808.

Kumar, S., Tamura, K., Jakobsen, I.B. and Nei, M. (2001). MEGA2: molecular evolutionary genetics analysis software. *Bioinformatics*. **17**:1244–1245.

Kupfer, M., Kuhnert P., Korczak, B.M., Peduzzi, R. and Dermarta, A. (2006). Genetic relationship of *Aeromonas* strain inferred from 16S rRNA, *gyrB* and *rpoB* gene sequences. *Int. J. Syst. Evol. Microbiol.* **56**: 2743-2751.

Laganowska, M. and Kaznowski, A. (2004). Restriction Fragment Length Polymorphism of 16S–23S rDNA Intergenic Spacer of *Aeromonas* spp. *Syst. Appl. Microbiol.* **27**(5): 549-557.

Lamy, B., Laurent, F., Verdier, I., Decousser, J.W., Lecaillon, E., Marchandin, H. and Kodjo, A. (2010). Accuracy of 6 commercial systems for identifying clinical *Aeromonas* isolates. *Diagn. Microbial. Infect. Dis*. **67**(1): 9-14.

Ludwig, W., Strunk, O., Klugbauer, S., Klugbauer, N., Weizenegger, M., Neumaier, J., Bachleitner, M. and Schleifer, K.H., (1998). Bacterial phylogeny based on comparative sequence analysis. *Electrophoresis*. **19**: 554-568.

Magni, M.V. (2010). Detection of bacteria, viruses, parasites and fungi: Bioterrorism prevention. *Springer*.

Martin-Carnahan, A.M. and Joseph S.W. (2005). Genus I. *Aeromonas* Stanier (1943). 213^{AL}. In: Brenner DJ, Krieg NR, Staley JT, Garrity GM (eds) Bergey's manual of systematic bacteriology, 2 nd edn, part B. *Springer*. **2**:557-578.

Martinez-Murcia, A., Benlloch, S. and Collins, D. (1992). Phylogenetic interrelationships of members of the genera *Aeromonas* and *Plesiomonas* as determined by 16S ribosomal DNA sequencing: lack of congruence with results of DNA-DNA hybridizations. *Int. J. Syst. Bacteriol.* **42**: 412-421

Minana-Galbis, D., Farfan, M., Fuste, M.C. and Loren, J.G. (2007). *Aeromonas bivalvium* sp. nov., isolated from bivalve mollusks. *Int. J. Syst. Evol. Microbiol*. **57**: 582-587.

Minana-Galbis, D., Urbizu-Serrano, A., Farfan, M., Fuste, M.C. and Loren, J.G. (2009). Phylogenetic analysis and identification of *Aeromonas* species based on sequencing of the cpn60 universal target. *Int. J. Syst. Evol. Microbiol*. **59**(8): 1976-1983.

Mount, D.W. (July, 2007). Using the Basic Local Alignment Search Tool (BLAST). *Cold Spring Harb. Protoc.* NY, USA. http://cshprotocols.cshlp.org/content/2007/7/pdb.top17.short. Retrieved November 12, 2013.

Nagar, V., Shashidhar, R. and Bandekar, J.R. (2012). Characterization of *Aeromonas* strains isolated from Indian foods using *rpoD* gene sequencing and whole cell protein analysis. *W. J. Microbiol. Biotechnol*. 1- 8.

Nhung, P.H., Hata, H., Ohkusu, K., Noda, M., Shah, M. M., Goto, K. and Ezaki, T. (2007). Use of the novel phylogenetic marker DnaJ and DNA–DNA hybridization to clarify interrelationships within the genus *Aeromonas*. *Int J Syst Evol Microbiol.* **57**: 1232-1237.

Nielsen, M. E., Hoi, L., Schmidt, A. S., Qian, D., Shimada, T., Shen, J.Y. and Larsen, J.L. (2001). Is *Aeromonas hydrophila* the dominant motile *Aeromonas* species that causes disease outbreaks in aquaculture production in the Zhejiang Province of China. *Diseases of aquatic organisms*. **46**(1): 23-29.

Ormen, O., Granum P.E., Lassen, J. and Figueras, M.J. (2005). Lack of agreement between biochemical and genetic identification of *Aeromonas* spp. *APMIS.* **113**: 203-207.

Parker J.L. and Shaw J.G. (2011). *Aeromonas* spp. clinical microbiology and disease. *J. Infect*. **62**: 109-118.

Rossello-Mora, R. and Amann, R. (2001). The species concept for prokaryotes. *FEMS Microbiol. Rev.* **25**: 39–67.

Saavedra, M.J., Figueras, M.J. and Martinez-Murcia, A.J. (2006). Updated phylogeny of the genus *Aeromonas*. *Int. J. Syst. Evol. Microbiol*. **56**: 2481–2487.

Saitou, N. and Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol*. **4**: 406–425.

Sarkar A., Saha, M. and Roy, P. (2012). Identification and Typing of *Aeromonas hydrophila* through 16S rDNA-PCR Fingerprinting. *J. Aquacult. Res. Dev*. **3**:146.

Shariff, M., Yusoff, F.M. and Gopinath, N. (1997). Aquaculture in Malaysia: Current Trends and Future Outlook. In Proceedings of Second International Seminar on Fisheries Science in Tropical Area. August 19-22. (p. 45-51),Tokyo, Japan.

Singh, V., Mani, I. and Chaudhary, D.K. (2012). Molecular Assessment of 16S-23S rDNA Internal Transcribed Spacer Length Polymorphism of *Aeromonas hydrophila*. *Adv. Microbiol.* **2**(2): 72-78.

Siti-Zahrah, A., Padilah, B., Azila, A., Rimatulhana, R. and Shahidan, H. (2005). Multiple *Streptococcal* species infection in cage-cultured red tilapia, but showing similar clinical signs. In *Proceedings of the Sixth Symposium on Diseases in Asian Aquaculture*. Colombo, Sri Lanka. **25**: 332-339.

Soler, L., Marco, F., Vila, J., Chacon, M.R., Guarro, J. and Figueras, M.J. (2003). Evaluation of two miniaturized systems, MicroScan W/A and BBL Crystal E/NF, for identification of clinical isolates of *Aeromonas* spp. *J Clin. Microbiol.* **41**: 5732–5734.

Stackebrandt, E. and Goebel, B.M. (1994). Taxonomic note: a place for DNA-DNA re-association and 16S rRNA sequence analysis in the present species definition in Bacteriology. *Int. J. Syst. Bacteriol*. **44**: 846-849.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. and Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance and maximum parsimony methods. *Mol. Biol. Evol.* **28**: 2731-2739.

Tan, C.K., (1998). Overview of Aquaculture in Malaysia. In Nagaraj and Singh (eds.) Aquaculture Practices in Malaysia. Occasional Publication No. 9. Kuala Lumpur: Malaysian Fisheries Society.

Teskeredzic, E., Strunjak-Perovic, I. and Coz-Rakovac, R. (2000). *Aeromonas hydrophila* isolated from wild freshwater fish in Croatia. *Vet. Res. Commun*. **24**(6): 371-377.

Vold, B. (1985). Structure and organization of genes for transfer ribonucleic acid in *Bacillus subtilis*. *Microbiol*. *Rev*. **49**: 71-80

Wang, G., Clark, C.G., Liu, C., Pucknell, C., Munro, C.K., Kruk, T.M. and Rodgers, F.G. (2003). Detection and characterization of the hemolysin genes in *Aeromonas hydrophila* and *Aeromonas sobria* by multiplex PCR. *J. Clin. Microbiol.* **41**(3): 1048-1054.