UNIVERSITI PUTRA MALAYSIA

GENETIC VARIATION BETWEEN AND WITHIN THREE VARIETIES OF DOMESTICATED TIGER BARB (*PUNTIUS TETRAZONA*) USING RAPD MARKERS

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By

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TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF ABBREVIATIONS

bp	Base-pair
DNA	Deoxyribonucleic Acid
DNTP	Dinucleotide triphosphate
cm	Centimetre
EDTA	Ethylenediamine tetra-acetic acid
EtBr	Ethidium bromide
g	Gram
HCL	Hydrocholoric acid
hr	Hour
kb	Kilobase
L	Litre
M	Molar
m	Meter
Min	Minutes
mM	Milimolar
MW	Molecular weight
mm	Milimetre
NaOH	Sodium hydroxide
ng	Nanogram
NaCl	Sodium chloride
rpm	Revolutions per minute
TBE	Tris-borate-EDTA
TE	Tris-EDTA buffer
UV	Ultra violet light
μg	Microgram
μl	Microlitre
V	Volt

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirements for the degree of Master of Science

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April 1999

Chairperson : Patimah Ismail, Ph.D.

Faculty : Medicine and Health Sciences

Tiger barb (*Puntius tetrazona*) is one of the popular ornamental fish species cultured and is a significant component of the aquarium fish industry in Malaysia. This study was conducted to assess genetic variation between and within three varieties of domesticated Tiger barb using random amplified polymorphic DNA (RAPD) markers. Genetic markers are important to selective breeding programmes and as a supportive research in order to minimise incorrect identification and stock contamination.

Fifty random individuals of each variety (normal, green and yellow) were obtained from Perak and Johor, which are the main producers of Tiger barb in

Malaysia. Twelve of 9-10 mer random primers with 50-77.7% of G+C content were used to generate the RAPD markers separable by agarose gel electrophoresis. total of 73 reproducible RAPD markers were found in the normal variety, 79 in the green variety and 84 in the yellow variety from Perak compared to 83 in the normal, 71 in the green and 74 in the yellow variety of reproducible RAPD markers with an average of 6-7 markers per primer.

Each variety of fish was found polymorphic with the normal variety identified as the most polymorphic (50.
the green was the least polymorphic (40.68%). Results of the RAPD analysis suggest that the low polymorphisms among individuals of the same variety indicate a high level of inbreeeding among the fish populations. The DNA fingerprints were assessed for genetic variability using Nei and Li's Similarity Index (SI). Bands were scored as present or absent for each variety and variation among varieties was quantified using the index of similarity.

The same variety from different states were grouped in the same cluster. The dendogram showed the closer genetic relationship between the normal and the green variety compared to the yellow variety. A breeding program can be formulated to increase the genetic variability within the varieties and populations using varieties with high SI and low SI. In this case, the green and yellow varieties should be crossed.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
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KEPELBAGAIAN GENETIK DI ANTARA DAN DI DALAM TIGA VARIETI TIGER BARB (*PUNTIUS TETRAZONA*) MENGGUNAKAN PETANDA RAPD

Oleh

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Tiger barb (*Puntius tetrazona*) merupakan salah satu daripada spesis ikan perhiasan popular dan satu komponen yang penting dalam industri ikan perhiasan di Malaysia. Kajian ini dilakukan untuk mengkaji kepelbagaian genetik di antara dan di kalangan tiga variasi ikan Tiger barb menggunakan petanda RAPD (Random Amplified Polymorphic DNA). Petanda genetik adalah penting bagi program pembiak-kacukan pemilihan dan sebagai kajian sokongan dalam meminimumkan salah pengenalpastian dan pencemaran stok.

Lima puluh individu telah dipilih secara rawak bagi setiap variasi (normal, hijau dan kuning) dari negeri Perak dan Johor yang mana merupakan pengeluar utama ikan Tiger barb di Malaysia. Dua belas primer berukuran 9-10 mer dengan kandungan G+C sebanyak 50-70% telah dipilih secara rawak untuk

membentuk petanda RAPD dan diasingkan menggunakan elektroforesis gel agarose. Sejumlah 73 petanda RAPD didapati pada variasi normal, 79 pada variasi hijau dan 84 pada variasi kuning dari Perak berbanding dengan 83 pada variasi normal, 71pada variasi hijau dan 74 pada variasi kuning dari Johor dengan purata 6-7 petanda RAPD bagi setiap primer. Setiap variasi ikan didapati polimorfik dengan variasi normal dikenalpasti mempunyai peratusan polimorfik tertinggi iaitu 50.57%, diikuti dengan variasi kuning (45.95%) dan variasi hijau mempunyai peratus polimorfik terendah (40.68%). Keputusan analisis RAPD mencadangkan nilai polimorfik yang rendah di kalangan individu dalam setiap variasi ikan menunjukkan paras pembiak-kacukan di kalangan ikan di dalam sesuatu populasi adalah tinggi. Petanda DNA dilakukan untuk mengenalpasti kepelbagaian genetik menggunakan index kesamaan Nei dan Li (1979). Petanda yang diperolehi adalah berdasarkan kepada ada atau tiada jalur DNA yang wujud untuk setiap variasi ikan dan kepelbagaian genetik di antara variasi dikira menggunakan indek kesamaan. Keputusan menunjukkan ikan dari variasi yang sama dari negeri yang berlainan dikelaskan di dalam kumpulan yang sama.

Dendogram yang diperolehi menunjukkan perhubungan genetik yang rapat di antara ikan Tiger barb variasi normal dan hijau berbanding variasi kuning. Satu program pembiak-kacukan boleh dilakukan untuk meningkatkan lagi kepelbagaian genetik di kalangan berbagai variasi ikan dan juga di dalam sesuatu stok populasi menggunakan variasi yang mempunyai indek kesamaan yang tinggi dan yang rendah. Daripada kajian ini, adalah dicadangkan variasi hijau dan kuning perlu dibiak-kacukkan.

CHAPTER I

INTRODUCTION

During the last four decades, there has been considerable growth and diversification in the international trade in ornamental fish. There is a growing popularity of fish keeping reflected as part of modern interior decoration. Their exotic colours and striking varied forms account for the interest in aquarium fish. Throughout the world, the ornamental fish trade and its associated industries have important economic status and are a major source of overseas income for areas of Africa, South America and South East Asia. The amount of world trade in aquarium fish is about RM18 billion in 1992 and the annual growth rate is about 10-15% (Andrews, 1992).

The aquarium fish industry in Malaysia is a developing industry and it gives good profit to the country. The production of aquarium fishes has been increasing year after year and this is proof that this industry has a good demand. In the Annual Fisheries Report 1995, aquarium fishes are classified into many popular groups that gave good income to the country. They are the cyprinids group (barbs/danio), cyprinids (carp/goldfish), anabantids (betta/gourami), poecilids (guppies/platies/swordtails/mollies), cichlids (mouth-breeder/egglayers), characins (tetras), osteoglossids and catfish (Anon, 1995).

Ferdouse, (1995) stated that aquarium or ornamental fish culture, was the most impressive sector which had risen by 84% between 1992 and 1993 and Johor had remained the main culture area. Over the past six years, aquarium fish products had increased steadily both in volume and value from RM 9,001,702.80 million in 1990 to RM 49,134,114.00 million in 1995 (Table 1). Aquarium fishes accounted for 10% of the total export value of aquaculture production.

Table 1: Production of aquarium fishes (number of fishes) and value from 1990-1995.

Year	Volume (Number of Fishes)	Value (RM)
1990	70,037,369	9,001,702.80
1991	88,584,841	14,501,714.82
1992	102,597,493	17,421,438.67
1993	188,871,525	33,653,755.29
1994	227,790,460	43,749,882.00
1995	253,075,201	49,134,114.00

Annual Fisheries Report (1991); (1995),
Department of Fisheries, Ministry of Agriculture, Malaysia.

The majority of aquarium fishes in the world market comes from Asian countries and Singapore is a main supplier (Bassler, 1995). The foreign investment in ornamental fish culture in Malaysia has been very encouraging; particularly from Singapore, which has became the trading centre for aquarium fish in Asia. Singaporean investors use Malaysia as a base to produce various species of freshwater aquarium fish. All exports are mainly through Singapore. However, due to increasing flight connections between Kuala Lumpur and other foreign markets direct exports from Malaysia has picked-up and is expected to accelerate in the near future. Malaysia has a great potential to become a major producer and exporter of aquarium fishes not only in Southeast Asia but also in the world market. In addition, Malaysia also has suitable land and support from the government, which helps in the development of the aquarium fish industry (Ferdouse, 1995). This is the time for Malaysia to produce new, attractive and large-scale ornamental fish species for the international market.

One of the popular aquarium fishes in Malaysia is Tiger barb and it has been cultured for its colour morphs. There is a considerable interest to utilise this species for the aquarium fish industry. Recognising their potential for aquarium fish industry, a comprehensive population study utilising both morphometrics and molecular techniques is currently being undertaken. It is to assess the variability of the species for the purpose of resource conservation and management and exploitation for the aquarium fish industry. A report by the Department of Fisheries showed that the export value of this fish had increased from RM17.5 million

(Malaysia, 1995) to RM61.22 million (309.10 million pieces) in 1997 (Thalathiah, 1998).

There is very little knowledge on the genetics of Tiger barb in Malaysia or any regard to the preservation and full utilisation of the fish's intrinsic genetic variability in the current Tiger barb breeding culture. This is likely to create problems which are often associated with poorly managed ornamental fish breeding programs. These problems include small founder populations, corrosion of genetic variability and inbreeding depression. In order to avoid these problems, a good knowledge of genetic relationships among the cultivated or domesticated varieties is fundamental.

A number of techniques developed over more than a decade has offered the opportunity to identify each individual or type of individuals in a species uniquely and unambiguously (Maniatis *et al.*, 1982). However, the genetic knowledge and molecular research of aquarium fish in Malaysia is limited although the number of researchers who use fish as model systems is increasing (Powers, 1989). Genetic analysis of organisms at the molecular level has become important and widely practised area of genetic science because of the limitation of morphological markers which is not accurate to distinguish between stocks or species compared to using molecular markers. Information on the level of genetic variability of Tiger barb stocks is useful for selective breeding programmes. By understanding the genetic structures of the stocks, the target variety can be produced easily. It is also a

supportive research in order to minimise incorrect identification and stock contamination. Thus it will help in the maintainance of the genetic diversity of the stocks.

A research programme entitled 'Development of DNA markers in Tiger barb (*Puntius tetrazona*)' was started at Universiti Putra Malaysia. This project was a Top-Down project under the Biotechnology Directorate, Ministry of Science, Technology and Environment, Malaysia. The aim of this project is to hasten the progress of the aquarium fish industry in Malaysia in order to produce new varieties of Tiger barb (*Puntius tetrazona*). The first phase in the project is to determine the DNA markers in this species by using Random Amplified Polymorphic DNA (RAPD) markers.

RAPD was chosen since it was shown to distinguish certain types of fish and colour mutants of Tiger barb by Dinesh *et al.*, (1992; 1993a). Although the research was new in aquarium fish in Malaysia, the logical starting point of research was the development of DNA markers in three varieties of Tiger barb. This chapter gives a review on the evolution of the research direction, which later led to the adoption of the current approach in genetic analysis. RAPD fingerprinting was selected because of many advantages compared to other molecular techniques. This method was less time consuming, simple, less costly, fast and allowed DNA typing without prior knowledge of DNA sequence (Hadrys *et al.*, 1992; Welsh and McClelland, 1994; Williams *et al.*, 1990).

There are many varieties of Tiger barb but only three varieties have been produced in large scale for the domestic and export markets. Johor and Perak are the main producers of aquarium fish as mentioned in Fisheries Annual Report by Fisheries Department (Malaysia, 1995; Thalathiah, 1998). In this research, the samples were taken from these two populations in order to determine the DNA markers of Tiger barb in domesticated stocks. The long-term objective of this project is to produce a new variety of Tiger barb from domesticated populations and not from wild populations. This is because they were produced by mass production from the breeding activity in the local farms and not caught from the wild.

RAPD markers can be used to identify broodstocks with high variabilities. This will increase genetic variability and thus maintain genetic diversity in these stocks. It can also be used to detect changes in genetic variation of Tiger barb stocks. For varieties that have very high levels of inbreeding those levels can be lowered by outcrossing and pedigree mating with stocks from other population. RAPD marker appears to be capable of contributing immensely to stock management through selective breeding and hybridisation programmes in the ornamental fish industry.

Not much work has been done on the genetic characterisation of local aquarium fish species through the use of molecular markers. In order to develop the aquarium fish industry in Malaysia, molecular genetic research should be strongly supported for the future management of fisheries resources. The accumulation of

molecular data on fish species in general can aid in fisheries research. From these research results and techniques the roles of the various genes in reproduction, growth and other aspects may be elucidated.

The objectives of this study were:

a) To develop DNA markers typing methods for Tiger barb varieties.
b) To determine the genetic differences between and within Tiger barb varieties based on RAPD markers.
c) To determine the differences of domesticated Tiger barb populations using RAPD markers.

As mentioned before, the main large-scale producers of Tiger barb for domestic and export market were Perak and Johor states. Recognizing their potential for the aquarium fish industry and to achieve the above objectives, three varieties of Tiger barb namely the normal, green and yellow varieties from Perak and Johor were used in this study. Fifty random samples from each variety were collected. Random primers were used to analyse DNA polymorphisms in order to detect the variety and population variabilities.

CHAPTER II

LITERATURE REVIEW

Morphology of Tiger barb

Tiger barb is one of the popular ornamental fish. The scientific name of Tiger barb is *Puntius tetrazona* or *Barbus tetrazona* and also *Capoeta tetrazona* (Axelrod, 1967). This species is also known as *Barbus sumatrana* on the basis of Bleeker's redescription from Sumatra (Axelrod and Schultz, 1990). They come under the family of Cyprinidae and genus Barbus. According to Mohsin and Ambak, (1983), Tiger barb was classified under:

Phylum	: Chordata
Class	: Pisces
Subclass	: Teleostei
Order	: Cypriniformes
Family	: Cyprinidae
Subfamily	: Cyprininae
Genus	: Puntius

It is locally known as 'pelampong jaring' (Mohsin and Ambak, 1983). They are tropical fish, which originated from Sumatra and Borneo (Axelrod, 1967;

and Nieuwenhuizen, 1991; Short, 1992; Mills, 1991). They can also be found in Thailand (Mohsin and Ambak, 1983; Dreyer and Keppler, 1996). According to Short (1992), the last name tetrazona refers to the four distinct zones or four black bars on its body that can reflect a deep bottle green colour when exposed to light (McInerny and Gerard, 1966). The body colour ranges from golden pink (Axelrod, 1967), bronze or tan (Mills, 1981), red brown fading to silver and orange brown pales (Short, 1992).

Tiger barb has deep and laterally compressed body without barbels (Dreyer and Keepler, 1966). The structure of the fins are follows, dorsal iv, 8; anal iv, 5; pectoral I, 13 or 14, pelvic I, 8; scale 23 (Axelrod and Schultz, 1990). The dorsal, anal, pelvic and pectoral fins are black at the base and red beyond the black bar.

They are highly coloured, can breed easily, adaptable to aquarium conditions (McInerny and Gerard, 1996), hardy and with flashy colours (Axelrod, 1967). According to Ramshorst and Nieuwenhuizen (1991) and Sandford (1995) they can reach a maximum length of 7 cm but most achieve about 5 cm. Tiger barbs are omnivorous fish which eat live and prepared food (Sandford, 1995). A mature female could release about 150-200 eggs (McInerny and Gerard, 1966) but they can also produce up to 600 eggs (Short, 1992). There are many varieties of Tiger barbs, namely albino, green (Mills, 1989; 1991), black (Sandford, 1995) and semi-translucent coral red colour (Gibbs, 1992).

Prospect of Tiger barb in the Aquarium Fish Industry

Over the past five years, fishery product exports increased steadily both in volume and value from RM577 million in 1989 to RM800 million in 1993. Between 1992 and 1993, aquarium or ornamental fish culture is the most impressive performing sector which rose by 84%. This is due to a rise in the exports of fisheries products which include aquarium fish that accounted for 10% of the export value and also because the use of old tin mines have increased substantially from 641 hectares in 1990 to 1,397 hectares in 1993 (Ferdouse, 1995).

German, Holland, France, Japan and USA are the main importer countries of aquarium fish. The value of aquarium fish depends on the quality because it is a live commodity. Only fish with perfect shape, attractive colour and healthy has market value. The largest markets for tropical aquarium fish are USA (US$ 100 million), EEC (US$93 million) and Japan (US$65 million) in 1992. For these three international markets Singapore is the largest supplier of aquarium fish. Seventy percent of the total export of Singapore originated from Malaysia (Bassler, 1995). Malaysia has suitable land for the development of aquaculture but labour shortage is a constraint. Potential investors may be compelled to consider high technology culture system for efficient, economical and viable aquaculture operation.

According to Annual Fisheries Report 1995, Tiger barb was classified into Cyprinids (Barb/Danio) group. Cyprinids have a potential for the export market. The main producers of Cyprinids were Perak and Johor. From 1992 to 1995, the volume