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PLOIDY DETECTION, GENETIC DIVERSITY, AND QUANTITATIVE TRAIT LOCI MAPPING IN MOLLY FISH (*Poecilia* spp.)

By

REMMY KEONG BUN POH

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

September 2012

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Doctor of Philosophy

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September 2012

Chairperson: Professor Siti Shapor Siraj, PhD

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A study was conducted to construct a preliminary linkage map for molly, *Poecilia* and to test association between markers and QTL traits. In linkage map construction, three major steps were involved. First is the development of mapping population. Background genetic information of the parental species such as occurrence of triploidy and genetic variability were crucial before any attempt to develop a mapping population. Triploid detection was carried out in this study by using highly polymorphic microsatellite (SSRs) primer pairs. Ten SSRs primer pairs could clearly differentiate among one, two and three alleles which could be used to infer different ploidy levels. The presence of two alleles indicates a diploid at that particular locus and evidence of three alleles could be inferred as triploid. If such association was direct, the occurrence of triploidy in molly fish sampled in this study was extremely low and negligible. The genetic diversity

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among the four species of live-bearer fishes namely *Xiphophorus maculatus, Xiphophorus helleri, Poecilia reticulata* and *Poecilia latipinna* were revealed using 15 polymorphic SSRs primer pairs. A total of 131 samples which comprised of these four species of live-bearers were collected in the middle of October 2009 from Aquatic International, Subang Jaya, Malaysia. Results showed that the clustering pattern of both *Xiphophorus* and *Poecilia* as revealed by these molecular markers seems to be in accordance with their taxonomy. In *P. latipinna,* the occurrence of inbreeding or outbreeding was absent as indicated by the level of heterozygosity. Majority of alleles were homozygous within *P. latipinna* and this seems to resemble the genome characteristic of a purebred fish. Purebred fish is an ideal parental material in any mapping population.

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A mapping population developed through backcross and testcross strategy was successfully attempted. A parallel study on the mode of inheritance of background body colour had also been initiated by evaluating body colour of progenies derived from crosses between two different colour variants of molly, [*P. latipinna* (non-black) and *P. sphenops* (black)]. Non-black body colour was found to be completely dominant over black and was not sex-linked. Multiple genes interaction which acted nonadditively was also found to be influencing this phenotypic trait. However, such interaction effect was restricted in crosses generated between genetically related fish.

The second step in linkage map construction is identification of polymorphism using molecular markers. A total of 142 SSRs primer pairs which includes those that were

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developed for *Xiphophorus maculatus* (123 primer pairs) and *Poecilia reticulata* (19 primer pairs) were screened and 95 primer pairs were able to be amplified on the DNA of molly, *Poecilia*. However, only 29 SSRs primer pairs were polymorphic when genotyped on 77 progenies derived from two backcrosses (n = 56) and a testcross (n = 21). The fish samples used for the development of mapping population were collected from Aquatic International Subang Jaya, Malaysia.

The final step is linkage analysis of polymorphic markers. Among the 29 polymorphic SSRs primer pairs identified, only 18 were informative and could be used to construct the linkage map. The constructed linkage map consists of four linkage groups covering a map size of 516.1 cM. One potential QTL, dorsal fin length was successfully associated between marker regions of Msd021, Msb069 and Msb068 on linkage group 2.

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The major drawback found in this study was low percentage of polymorphic SSRs primer pairs. Such condition not only greatly restricted the number of markers that could be positioned on the linkage map but also minimizes the number of linkage group that could be generated. Map enrichment effort using both amplified fragment length polymorphism (AFLP) markers and development of new SSRs primer pairs for this species are strongly suggested.

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Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PENGESANAN PLOIDI, KEPELBAGAIAN GENETIK, DAN PEMETAAN LOKUS TRAIT KUANTITATIF IKAN MOLLY (*Poecilia* spp.)

Oleh

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Satu kajian dijalankan untuk membina rantaian peta awalan molly, *Poecilia* dan mengenalpasti sama ada terdapat gabungan antara penanda dengan ciri QTL. Pembinaan pemetaan jaringan melibatkan tiga langkah utama. Pertama ialah pembangunan populasi pemetaan. Kajian asas genetik tentang spesies induk seperti kewujudan 'triploid' dan variasi genetik adalah penting sebelum percubaan untuk membangunkan populasi pemetaan. Pengesanan 'triploid' dalam kajian ini dijalankan dengan menggunakan penanda mikrosatelit (SSRs) yang polimofik. Sepuluh penanda SSRs ini dapat membezakan antara satu, dua dan tiga alel boleh digunakan untuk menghasilkan tahap 'ploidy' yang berbeza. Kemunculan dua alel menunjukkan 'diploid' di lokus yang tertentu dan bukti tiga alel boleh digunakan untuk membuat kesimpulan sebagai

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'triploid'. Jika hubungan ini boleh digunakan secara terus, kewujudan 'triploid' dalam ikan molly yang di sampel dalam kajian ini adalah berada pada tahap yang rendah dan boleh diabaikan. Kepelbagaian genetik antara keempat-empat ikan 'life-bearer' iaitu *Xiphophorus maculatus, Xiphophorus helleri, Poecilia reticulata* dan *Poecilia latipinna* didedahkan dengan 15 penanda SSRs yang polimofik. Sejumlah 131 sampel yang terdiri daripada empat spesies 'live-bearer' telah dikutip pada pertengahan Oktober 2009 dari Akuatik Antarabangsa, Subang Jaya, Malaysia. Keputusan menunjukkan corak kelompok untuk kedua-dua *Xiphophorus* dan *Poecilia* seolah-olah menyerupai pengelompokan berdasarkan taksonominya. *P. latipinna,* tidak menunjukkan keadaan pembiakbakaan dalaman ataupun luaran sepertimana yang ditunjukkan oleh tahap heterozigositi. Kebanyakkan alel dalam *P. latipinna* adalah homozigot dan ini menyerupai ciri-ciri genom ikan ras tulen. Ikan ras tulen merupakan induk yang paling sesuai dalam membangunkan populasi pemetaan.

Populasi pemetaan yang dibangunkan melalui strategi pembiakbakaan kacukan balik dan kacukan uji telah berjaya dilaksanakan. Kajian selari pada mod pewarisan warna belakang latar badan telah dilaksanakan dengan menilai warna badan anak ikan yang dihasilkan melalui kacukan antara dua ikan molly yang mempunyai variasi warna badan yang berlainan, [*P. latipinna* (bukan hitam) dan *P. sphenops* (hitam)]. Bukan hitam didapati adalah dominan kepada hitam dan pewarisan ini tidak berkaitan seks. Interaksi pelbagai gen secara 'nonadditive' juga didapati mempengaruhi ciri fenotip ini. Namun begitu, kesan interaksi ini hanya boleh dikesan di antara kacukan yang mempunyai pertalian genetik.

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Langkah kedua dalam pembinaan pemetaan genetik adalah mengenalpasti penanda yang polimorfik. Sejumlah 142 penanda SSRs yang dibangunkan untuk *Xiphophorus maculatus* (123 penanda) dan *Poecilia reticulata* (19 penanda) telah diperiksa dan hanya 95 yang menunjukan amplifikasi pada DNA molly, *Poecilia*. Namun begitu, hanya 29 penanda SSRs yang polimofik semasa digenotip pada 77 progeni yang terhasil daripada kacukan balik (n = 56) dan kacukan uji (n = 21). Sampel ikan yang digunakan dalam pembangunan populasi pemetaan dikutip daripada Akuatik Antarabangsa, Subang Jaya, Malaysia.

Langkah akhir ialah analisis jaringan penanda yang polimorfik. Antara 29 penanda SSRs yang polimofik, hanya 18 yang berinformasi untuk digunakan dalam membina peta jaringan genetik. Peta jaringan genetik yang dibina terdiri daripada empat kumpulan jaringan yang merangkumi jarak peta 516.1 cM. Satu QTL yang berpotensi, panjang sirip dorsal berjaya dikaitkan dengan penanda Msd021, Msb069 dan Msb068 pada peta jaringan nombor 2.

Kelemahan utama yang dikesan daripada kajian ini adalah peratusan yang rendah pada penanda SSRs yang polimofik. Keadaan ini bukan sahaja menghadkan bilangan penanda yang boleh dipetakan malah menghadkan bilangan peta jaringan yang boleh dijana. Usaha untuk menghasilkan peta jaringan yang padat dengan menggunakan penanda 'amplified fragment length polymorphism (AFLP)' dan pembangunan penanda SSRs yang baru untuk spesies ini adalah disyorkan.

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I certify that a Thesis Examination Committee has met on 6 September 2012 to conduct the final examination of Remmy Keong Bun Poh on his thesis entitled "Ploidy Detection, Genetic Diversity, and Quantitative Trait Loci Mapping in Molly Fish (*Poecilia* spp.)" in accordance with the Universities and University College Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The committee recommends that the student be awarded the Doctor of Philosophy.

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DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institution.

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LIST OF ABBREVIATIONS

µl	Microlitre
1X	One time
AFLP	Amplified fragment length polymorphism
BC	Backcross
bp	Base pair
cm	Centimeter
ddH_20	Double distilled water
dNTP	Deoxynucleotide triphosphate
F_1	First filial generation
F_2	Second filial generation
LOD	Logarithm of odds
mg	Milligram
min	Minute
ml	Millilitre
mM	Millimolar
n	Number
PCR	Polymerase chain reaction
QTL	Quantitative trait loci
RAPD	Random amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
rpm	Revolution per minute

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s	Second
SNP	Single nucleotide polymorphism
SSRs	Microsatellites
TBE	Tris-borate EDTA buffer
U	Unit
USA	United States of America

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CHAPTER 1

INTRODUCTION

An annual report by the Department of Fishery Malaysia had stated that ornamental fish production had increased from RM 72 million in the year 2000 to RM 458 million in the year 2008 (Che Ayub, 2010). Such revenue had contributed to a huge portion of income in Malaysian fish industry. The ornamental fish production was carried out extensively to meet the high demand both for local hobbyist and foreign export. Ornamental fish such as the molly fish is popular among aquarist because they are relatively easy to maintain in captivity, beautiful in appearance and easy to breed. According to a separate report by Department of Fishery Malaysia (DOF, 2011), molly fish is one the major trading ornamental fish species in Malaysia.

Mollies belong to the genus *Poecilia* and originate from Mexico and USA (Hankinson and Ptacek, 2008), are widely distributed around the globe, including Malaysia. Three species of mollies are available in the local market, namely *Poecilia latipinna, Poecilia velifera* and *Poecilia sphenops.* According to Ptacek and Breden (1998), both *P. latipinna* and *P. velifera* are classified in the group of *P. latipinna* complex while *P. sphenops* is in the group of *P. sphenops* complex. *P. latipinna* and *P. velifera* are biologically and morphologically similar to each other, having an enlarged dorsal fin that is used during courtship display. On the other hand, *P. sphenops* has tipped caudal fin at both ends which resemble a crescent moon.

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Mollies are popular as a fish pet due to the attractiveness of their body colour and relative ease to maintain in an aquarium. A vast number of studies had been carried out in this fish species focusing on chromosome karyotyping (Haaf and Schmid, 1984), occurrence of triploidy (Lampert *et al.,* 2008), mating behaviour (Hankison and Ptacek, 2008; Ptacek, 2002; Marley *et al.,* 1997), morphology differences (Hankinson and Ptacek, 2008; Ptacek, 2002), phylogenetics and population structure (Hankinson and Ptacek, 2008; Lampert *et al.,* 2006; Ptacek and Brenden, 1998). To date there is no information and report to provide a guide on gene trait association on this species. An approach to understand gene trait relationship is through construction of a genetic map.

A genetic map will show the gene position or DNA sequences on a chromosome. Studying a single gene sequence would be more beneficial if the position of other genes involved in the whole genome of the species is known. This is especially for traits such as melanophore positions (Tripathi *et al.*, 2009b) and dorsal fin length (Ptacek, 2002) which are polygenically inherited. In other words, one or more than one gene might be responsible for the appearance of a single trait. In order to have a better understanding on all the potential gene sequences involved in determining a trait, construction of a Quantitative Trait Loci (QTL) map which display all loci having major, minor or suggestive effect are essential.

A mapping population is required for construction of a linkage map. Parents selected for the mapping population must differ for one or more trait(s) of interest. When developing a mapping population, fertility of the fish to be advanced for breeding is another

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important aspect to be considered. Triploidy occurrence had been reported in Amazon mollies both in captivity (Rasch *et al.,* 1965; Schultz and Kallman, 1968) and in the wild (Balsano *et al.,* 1972; Turner *et al.,* 1983). Triploid fish is usually sterile (Schultz and Kallman, 1968; Nanda *et al.,* 1995). A preliminary screening for triploid is essential and should be carried out to ensure the fertility of all the progenies. Methods for triploid detection should be cost-effective, simple and accurate (Tiwary *et al.,* 2004). Background genetic information of any fish species either in their natural environment or captivity is crucial to assists in selection of starting material in any breeding programme. For instance, information such as heterozygosity level could help to identify whether a species is experiencing inbreeding or outbreeding.

Different types of markers had been used for construction of linkage as well as QTL map in fish. Linkage map constructed by Tripathi *et al.* (2009a) on guppy (*Poecilia reticulata*) were largely based on Single Nucleotide Polymorphism (SNP) markers. Microsatellite (SSRs) markers are another popular option and have been widely utilized for construction of linkage map such as in *Xiphophorus* sp (Walter *et al.,* 2004; Kazianis *et al.,* 2004). In constructing a linkage map, it is advisable to use markers (SSRs) developed specially for that particular species to avoid problems such as unspecific priming and low polymorphism. However, developing these markers is laborious and costly. An alternative would be cross-species amplification utilizing SSRs markers that have already been developed for a closely related species. These markers will, however, have to be subjected to amplification trial and polymorphism screening. The information

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on the genetic diversity especially on the relationship between closely-related species is important in selection of SSRs markers to be cross-amplified.

Hence, this study is conducted to construct a linkage map for molly, *Poecilia* utilizing microsatellite markers. The linkage map will indicate the position and relative genetic distances between the markers. The next step will be QTL mapping, which is associating the potential markers with the traits. The objectives of this study were as follows:

1. To detect ploidy level in molly, *Poecilia,* by using microsatellite loci.
2. To identify the genetic diversity among live-bearer fishes (molly, platy, swordtail and guppy) using cross-amplification microsatellite markers developed for *Xiphophorus* species.
3. To study polygenic inheritance of background body colour in hybrids of *Poecilia latipinna* and *Poecilia sphenops.*
4. To construct a preliminary linkage map for molly, *Poecilia*.
5. To test association between markers and QTL traits in molly, *Poecilia*.

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