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THESIS

GENETIC DIVERSITY OF HATCHERY AND WILD POPULATIONS OF *Cirrhinus cirrhosus* (Bloch, 1795) IN MYANMAR

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Ohnmar Aung 2009: Genetic Diversity of Hatchery and Wild Populations of *Cirrhinus cirrhosus* (Bloch, 1795) in Myanmar. Master of Science (Aquaculture), Major Field: Aquaculture, Department of Aquaculture. Thesis Advisor: Associate Professor Supawadee Poompuang, Ph.D. 70 pages.

Six microsatellite loci from *Barbodes gonionotus*, *Cyprinus carpio* and *Labeo rohita* were used to reveal genetic difference from five wild and five domesticated stocks of mrigal, *Cirrhinus cirrhosus* in Myanmar and one hatchery stock in Vietnam. Two hundred and eleven wild samples were collected from the Ayeyarwaddy and Thanlwin River systems and 216 samples were collected from five hatcheries stations under the Department of Fisheries. Forty three samples from a hatchery in Vietnam were used for comparative purposes.

All microsatellite loci examined displayed high levels of polymorphism in all populations. A total of 112 alleles were detected at six loci, ranging from 10 alleles at locus *Bgon22* to 29 alleles at locus *MFW17*. For Myanmar populations, mean number of allelic richness (A_r) in the hatchery samples ranged from 5.24 to 8.41, and in the wild samples ranged from 5.34 to 8.52. Vietnam hatchery stock, however, exhibited lower value of allelic richness at 2.31. Expected heterozygosities ranged from 0.56 to 0.79 in the wild samples and from 0.69 to 0.79 in the hatchery populations. Six populations showed significant deviations from H-W equilibrium for all loci after sequential Bonferroni adjustment.

The estimate of F_{ST} value (0.134) indicated high levels of differentiation among populations. The MDS plot based on pairwise F_{ST} values displayed four groups of Myanmar samples and a distinct group of VN samples which separated from the rest. Results of clustering analysis were in agreement with the MDS analysis. AMOVA analysis revealed no genetic differentiation between wild and hatchery samples from Myanmar (F_{CT} = 0.000; P>0.05), and 97.15% of genetic variation could be attributed to within populations variation (F_{ST} = 0.028; P<0.05). In conclusion, Myanmar populations exhibited relative high genetic diversity and cryptic population genetic structure was found in the hatchery populations. The population genetic information obtained in this study should prove useful for aquaculture management, breeding programs and enhancement programs of mrigal populations in Myanmar.

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Student's signature Thesis Advisor's signature

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GENETIC DIVERSITY OF HATCHERY AND WILD POPULATIONS OF *Cirrhinus cirrhosus* (Bloch, 1795) IN MYANMAR

INTRODUCTION

Cirrhinus cirrhosus (Hamilton 1822), popularly known as mrigal, is a freshwater species of the family Cyprinidae. It is widely distributed across the Indian sub-continent, encompassing countries such as Pakistan, India, Bangladesh, Nepal and Myanmar. The species also has been translocated widely in Asia for aquaculture purposes (Vidthayanon *et al.* 2005).

Mrigal is a very important protein source for rural communities in many countries. The relatively higher growth rate of mrigal, coupled with its compatibility with other carps, has helped in establishing this species as one of the principal component species in pond polyculture culture system. Global aquaculture production has been expanded rapidly up to 1996, and reached the peak of 552,000 tons in 2000 and 445,000 tons in 2001 (FAO 2009). Major producers include India, Bangladesh and Myanmar.

Mrigal aquaculture in Myanmar has a long history, dating back to early 1970s. The Department of Fisheries of Myanmar has chosen mrigal as a target species for aquaculture in order to meet food security policy of the country and breeding plans for mrigal have been established since 1971. Recently, Myanmar has developed export markets for mrigal and aquaculture of this species is on an increase. In addition, since the 19th century, mrigal have been restocked into a number of water bodies including

rivers, lakes and reservoirs to replenish the depleted wild populations and enhance the fisheries of these water bodies.

The above practices have brought to the forefront regarding the genetic management of mrigal in aquaculture and stock enhancement in Myanmar. Firstly, long-term management of aquaculture of mrigal in Myanmar would be compromised without a proper genetic management plan. In this regard, information on levels of genetic variability within and among broodstock populations permits fish breeders to avoid potential detrimental effects of inbreeding and other genetic changes from one generation to another (Gjedrem 1992). Also, genetic markers can be used to assist establishment of base-line stock, including family lines for selection purposes (Cross *et al.* 2000).

Secondly, genetic variability is pivotal to maintaining the capability of restocked fish to adapt to a changing environment (Avise 1994). However, there have been documented cases in many fish species of genetic changes and loss of genetic variability in hatchery-reared stocks, and also resulting in alteration of genetic diversity of their wild counterparts due to interbreeding with escapees of hatchery-reared stocks (Crozier 1993; Clifford *et al.* 1998) or those used for restocking (Bentsen 1991; Hindar *et al.* 1991).

Molecular genetic markers have been proven useful in addressing questions relating to levels of genetic diversity and structure of natural populations, which in turn provide useful information for development of management plans for cultured species. The lack of systematic genetic surveys to ascertain the genetic variability within and between populations of mrigal prompted the present study, the results of which may help in the design of a broodstock management strategy and subsequent

application to future aquaculture production and stock enhancement strategies (Ryman and Laikre 1991).

OBJECTIVES

The overall goal of this study was to obtain base-line information on genetic status of wild and hatchery populations of mrigal. This information is needed not only for long term management of sustainable fisheries resources but also for establishment of selective breeding programs for fisheries communities in Myanmar.

Specific objectives

The specific objectives of this study are:

1) To investingate levels of genetic diversity of hatchery and wild populations of mrigal in Myanmar using microsatellite DNA markers, and
2) To provide recommendations for on the management and sustainable use of genetic resources of mrigal in Myanmar based on the findings in objective 1.

LITERATURE REVIEW

1. Biology and distribution of *Cirrhinus cirrhosus*

Cirrhinus cirrhosus, commonly referred to as mrigal, is a freshwater fish of the family Cyprinidae (Figure1). It inhabits rivers and floodplains of large rivers such as the Ganges and the Ayeyarwaddy systems (Vidthayanon *et al.* 2005). Recently, mrigal is translocated to many countries for aquaculture purposes, such as Sri Lanka, Vietnam, China, Mauritius, Japan, Malaysia, Philippines and the former USSR (FAO 2009).

Current taxonomic status of mrigal is confusing. According to Fish Base, a number of species in the genus *Cirrhinus* are synonyms of *C. cirrhosus*, such as *C. mrigala* (a widely used scientific name of mrigal) and *C. blochii, C. chaudhryi*, *C. horai, C. cirrhosa, C. cuvierii* , and *C. cauverii*. This revision was supported by Roberts (1997) and Kottelat (2001). Vidthayanon *et al.* (2005) recorded the species in Myanmar as *C. cirrhosus.*

This species includes other six primary species: common carp *Cyprinus carpio*, grass carp *Ctenophalyngodon idella*, silver carp *Hypophthalmichthys molitrix*, bighead carp *Aristichthys nobilis*, catla carp *Catla catla*, and rohu, *Labeo rohita* (Hulata 1995).

Figure 1 Mrigal *Cirrhinus cirrhosus*

Mrigal is bottom feeder herbivore. In natural waters, the fish shows a very rapid growth rate in the first four years, followed by a period of slow growth in the next three year. The growth rate thereafter are slower (Salam *et al.* 2002). It can survive up to 12 years and have a maximum size of 12.7 kg and 992 mm long after two years. Mrigal can lay eggs at the end of swift-moving streams with a sandy or clay bottom at the depth of 50-100 cm. The fecundity of mrigal ranged from 100,000 to 200,000 eggs/kg of female bodyweight. Jhingran and Pullin (1985) recorded that maximum number of eggs released by a female (weight 4.76 kg) was 1,164,000. The females can be used for spawning at 2 years old but the males can attain the first maturity at the end of the first year in artificial fish ponds.

2. History of mrigal aquaculture

Mrigal (Nga-Gyin-Phyu in Burmese) is widely cultured in India, Bangladesh, Myanmar and Southeast Asian countries. The traditional culture of the species was restricted to eastern parts of India until the 1950s (FAO 2009). However, mrigal become one of the major culture carp species after the successful of artificial propagation technology in the 1960s. Mrigal can grow fast, coupled with its compatibility with other species, have made it a major component in the polyculture system which is popular in many rural areas.

Mrigal has become a major cultured species in rural Asia. Trends in aquaculture production of mrigal are given in Figure 2. Global aquaculture production has expanded rapidly up to 1996, and reached the peak of 552,000 tons in 2000 and 445,000 tons in 2001 (FAO 2009). Major producers include India, Bangladesh and Myanmar.

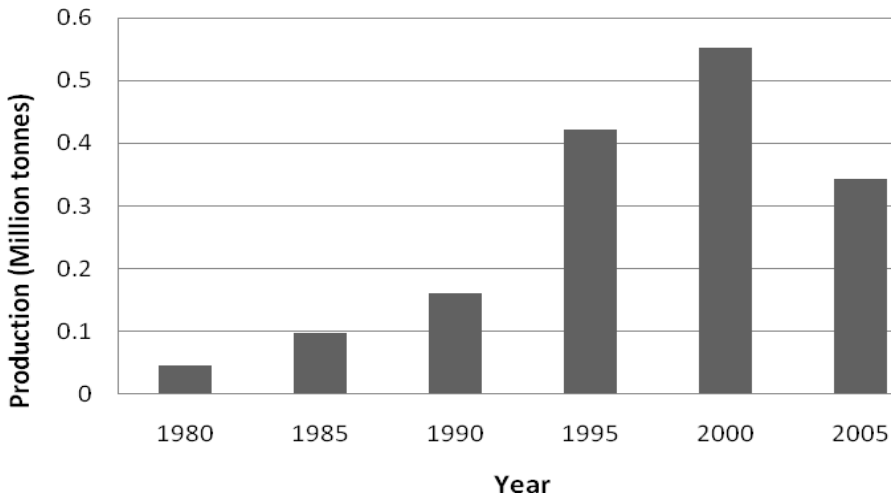

Figure 2. Trends in global mrigal aquaculture production

Source: FAO (2009)

Myanmar has rich inland water resources covering 8.1 million ha, of which 1.3 million ha are permanent and the remainders are seasonally inundated floodplain (RAP-FAO 2003). It is the natural inhabitant of the freshwater bodies of Ayeyarwaddy, Chindwin, Sittaung, and Thanlwin rivers. Mrigal was first reported in 1829 from Ayeyarwaddy river in Bhamo township and Yangon city (Banarescu 1977).

Early culture of mrigal was dependent on fry captured from natural river systems and inundated floodplains or leasable fisheries, located in Thrawaw, Hinsata, Shwe Daung, Pyae townships in lower Burma and Mandalay district and in Amarapura (Mandalay) around Myit Ngae River and Sintgu areas. During south-west monsoon season, the inundated floodplains serve as migration, breeding and nursery grounds of most freshwater fish and prawn species. The peak season for capturing fish migrating off the floodplain is from May to July (Win and Lay 2003).

In 1971, induced breeding of mrigal was successful to support fry supply for farmers. Breeding of mrigal in fisheries industries is mainly performed during July to September which corresponds to the Southwest monsoon in Asia. Initially, founder populations of mrigal at all of the fisheries stations were taken from the difference branches of Ayeyarwaddy river basin and leasable fisheries during the raining season without genetic consideration and the number of founding individuals was not known. Recently, the Department of Fisheries has established a restocking program to replenish the natural resources of mrigal in the open waters like rivers, dams, reservoirs, lakes and impoundments (Fisheries Department 2007).

3. Population genetics

Population genetics encompasses the description of the distribution of genetic variation within and between populations based on the assumptions of Hardy-Weinberg equilibrium, thereby providing indirect information on population isolation or structure. Hardy-Weinberg principle is the basic concept of population genetics. Under this principle, it is assumed that the organism is diploid, reproduction is sexual, generations are non-overlapping, the gene under consideration has two alleles, allele frequencies are identical in males and females, mating is random, migration is negligible, mutation can be ignored, and the population size is large (Hartl and Clark 1997). Hardy-Weinberg equilibrium estimates expected genotype frequencies using allele frequencies that are constant from one generation to the next generation in population without disturbing processes.

More broadly, population genetics provides information on genetic diversity that can be used to measure levels of inbreeding, gene flow, population subdivision, and migration rates within and among populations (Weir 1990; Page and Holmes 1998). Populations may exhibit different kinds of genetic structure, and determining the appropriate structure is probably the first step in understanding population biology (Baverstock and Moritz 1996b). In the most extreme case of no genetic variation, a species may consist of only a single population unit (i.e. panmixia), or many isolated subpopulations but no genetic differences. However, genetic subdivision of species into multiple populations is common (Johnson 2000). A species can consist of a series of subpopulations in which genes are likely to be exchanged with adjacent populations (the stepping-stone model), or each subpopulation is equally likely to exchange genes with any other subpopulation (the islands model), or a series of isolated subpopulations within which individuals exchange genes but the more isolated the subpopulations are, the less likelihood of gene flow between them (the isolation-by-distance model). These three different models of population structure result in different patterns of genetic differentiation within and between geographic localities (Richardson *et al.* 1986; Slatkin 1987).

4. Genetic diversity

Genetic diversity refers to the variety of genes or hereditary units. Genetic diversity can be defined by the amount and distribution of genetic variation within and among populations as determined by the effects of mutation, natural selection, genetic drift and gene flow (Frankham *et al.* 2002) and (Hartl and Clark 1997). The differences are the result of evolutionary process that reflects adaptations of individuals or populations (Ayala 1982). Genetic diversity is estimated based on the variation of alleles in an individual and difference of allele frequencies in the populations.

4.1 Genetic variation within populations

Genetic variation within populations determines the ability of a species to survive in changing environment. Population size is the single most important factors to sustain a high level of genetic variation within a population (Frankel and Soule 1981). Reduction in population size at the time of fragmentation creates genetic bottlenecks. Differential survival of progeny and non-random mating can lead to low levels of genetic variation which can have a direct impact on the availability of favorable genes and increase the risk of inbreeding depression (Lind *et al.* 2008).

The amount of genetic variation within populations can be measured by the parameters such as allelic diversity or allelic richness, effective number of alleles and the observed and expected heterozygosities (Allendorf and Luikart 2006). Allelic diversity is measured from the number of alleles at a locus in a population. The number of alleles in a sample, however, is affected by the size of a sample because large samples are expected to contain more alleles than the small samples. Leberg (2002) developed a statistical method to produce unbiased estimates of allelic richness.

Effective number of allele (a_e) describes change in allele variance for each locus by the formula, $\frac{1}{\sum p_i^2}$, where p is the ith allele frequency (Ciftci and Okumus 2003).

Observed heterozygosity (H_o) is a proportion of heterozygotes averaged across loci and individuals. Expected heterozygosity (H_e) is estimated from observed frequencies of alleles assuming the population is in Hardy-Weinberg equilibrium. The level of expected heterozygosity (H_e) for a particular locus is calculated by $H_e = 1 - \sum_{i=1}^{k} p_i^2$ where p, is the frequency of the ith allele in a population and k is the number of allele (Ciftci and Okumus 2003).

4.1.1 Inbreeding and Wahlund effect

Inbreeding refers to mating between related individuals. Inbreeding affects the genetic variability in populations which can bring one or more identical recessive genes leading to a situation of reduction of fitness. The offspring of related parents have more homozygotes at one or more loci than unrelated parents. Inbreeding results in excess of homozygotes relative to Hardy-Weinberg expectations. Inbred populations exhibit decline in growth rate, survival rate, and fecundity, as well as smaller adult size and depressed production. In hatchery stocks of fish, reductions of survival and reproduction are highly associated with inbreeding (Vrijenhoek 1998).

The mixing of two populations with different allele frequencies also results in an excess of homozygotes or a deficiency of heterozygotes, even if Hardy-Weinberg proportions exist within each population. This is known as Wahlund effect (Hedrick 2005). The reduction in heterozygosity can be measured using *F*-statistics (Wright 1965). When there is no deficit of heterozygosity, $F_{IS} = 0$.

4.1.2 Founder effect and population bottlenecks

Founder effect is a phenomenon which a population grows from a fewer individuals. A bottleneck is caused by a suddenly restriction in population size as can occur when a species is overexploited or populations are isolated as a result of habitat fragmentation (Frankham *et al.* 2002). Only a small number of individuals survive in the consecutive generations to continue the existence of the population. Both founder effect and bottleneck can lead to changes in allele frequencies which different from the ancestral population at a certain time or place. These situations can result in fewer alleles, reduction in the number of polymorphic loci and heterozygosity. In aquaculture and restocking programs, founder effects are resulted from the use of small number of individuals as founder broodstock population. In addition, founder effect and subsequent bottlenecks are the major

causes that increase inbreeding in hatchery stocks of small population size (Shikano *et al.* 2003).

4.1.3 Genetic drift and effective population size

Genetic drift refers to random change in allele frequencies due to sampling errors. Genetic drift is directly related to population size. In small populations, genetic drift can have large effects on genetic variation. The effective population size of a population (N_e) is the size of an ideal population which meets all the Hardy-Weinberg assumptions that would lose heterozygosity at a rate equal to that of the observed population. The effective population size is a theoretical measure of how many individuals are contributing their genes to the next generation. In general, N_e is less than the actual number of animals present in the population. There are several factors that cause reduction of N_e, including fluctuating population size, unequal breeding sex ratio, and variation of family size. If N_e is small, inbreeding and loss of allelic frequencies as a result of genetic drift increase. A number of studies suggested that reductions in N_e had adverse effects on the gene pools of hatchery stocks (Ryman and Utter 1987). In fish, N_e can be much smaller than the actual population size due to high fecundity of females and minimal numbers of breeding individuals can produce large sized populations (Lakra *et al.* 2007).

4.1.4 Linkage disequilibrium

When the genetic variation at two or more loci is considered simultaneously, the allele frequencies are insufficient to describe the genetic variation. Instead, multi-locus gamete frequencies must be utilized because association of alleles within gametes may occur (Hedrick 2005). However, various factors can cause linkage disequilibrium including genetic drift, mutation, inbreeding and gene flow or hybridization between two populations. Linkage disequilibrium is strong in small population as a result of random genetic drift. It corresponds to the difference between the expected and the observed genotypic frequencies. The range of linkage

disequilibrium is 0 to 1. The result is zero if no other processes are acting except recombination. This can result in changes in allele frequency and loss of alleles from populations.

4.2 Genetic differentiation between populations

The genetic differentiation between populations is determined by dispersal abilities in populations. The subpopulation has different survival and death rate due to natural selection. Genetic differentiation can be influenced by a number of evolutionary forces and their interaction that act on natural populations including migration, random genetic drift and mutation (Hartl and Clark 1997). Genetic differentiation can be quantified based on genetic distance, *F*-statistics and the amount of gene flow between populations.

4.2.1 Genetic distance

Genetic distance is a measure of the dissimilarity or similarity of genes between species or populations (Nei 1973). Genetic similarity between two populations implies that they recently separated into two populations or gene flow occurred between them. Differences between two populations suggest that they have been isolated for a long time and there have been no gene flow between them. Genetic drift also can generate large differences between populations (Hedrick 2005). Small estimations of distance may indicate population substructure as a result of two different processes. First, there is a reduced amount of gene flow between populations. Second, the populations are completely isolated but have only been separated for a short period of time.

4.2.2 *F-statistics*

The most important approach for measurement of the amount of population differentiation is *F* coefficients (Wright's *F*-statistic). Wright (1969) developed the approach to partition the genetic variation in a subpopulation to provide a description of differentiation. *F*-statistics can be thought of as a measure of the correlation of alleles within individuals and are related to inbreeding coefficients. An inbreeding coefficient is a measure of the nonrandom association of alleles within an individual. The *F*- statistics describe the amount of inbreeding-like effects within subpopulations F_{IS}, among subpopulations F_{ST}, and within the entire population F_{IT}.

(1) F_{IS} is also known as the fixation index, measures the deviation from Hardy Weinberg expectation within subpopulations to the reduction of heterozygosity due to non-random mating in subpopulations.

$F_{IS} = \frac{H_S - H_O}{H_S}$, where H_O is the average observed heterozygosity within a subpopulation, and H_S is the average expected heterozygosity within subpopulations. The value of F_{IS} ranges from -1 to +1. Negative (low) values indicate heterozygote excess (outbreeding) and positive (high) values indicate heterozygote deficiency (inbreeding) as compared to H-W expectations.

(2) F_{ST} measures the amount of genetic differentiation over subpopulations and is always positive. The value displays the reduction in total expected heterozygosity in subpopulations as compared to the average expected heterozygosity from the total population.

$F_{ST} = \frac{H_T - H_S}{H_T}$, where H_T is the average expected heterozygosity in total population and H_S is the average observed heterozygosity in subpopulation.

(3) F_{IT} measures the correlation of alleles for the entire population which is a combination of both the within and among subpopulation effects, and can be estimated from:

$F_{IT} = \frac{H_T - H_O}{H_T}$, where H_T is the average expected heterozygosity among subpopulations.

4.2.3 *Gene flow and population differentiation*

Gene flow or migration is the important source of genetic variation. Limited gene flow not only causes higher genetic differentiation among populations but also lower the genetic diversity of each population. The level of gene flow can be estimated from value, $F_{ST} = \frac{1}{1 + 4N_e m}$, where N_e is the effective population size and m is the fraction of migrants per generation (Wright 1969). High numbers of migrants results in low F_{ST} values. If there is no gene flow between two subpopulations, the F_{ST} will reach 1.

5. Application of microsatellites for population genetic analysis

Molecular genetics has proven useful in assessing the extent and patterns of population subdivision as well as for investigation of the forces that change population structure (Avise 1994; Hillis *et al.* 1996). The general advantages of molecular markers for the study of population structure are their ready availability, genetic simplicity, comparability across taxa, and ease of use with population genetic models (Johnson 2000).

Various molecular genetic techniques have been employed to address population related issues, including allozyme electrophoresis (Richardson *et al.* 1986;

Avise 1994; Shaklee and Benzen 1998) and DNA-based markers, e.g. nucleotide data (Hillis *et al.* 1996) and microsatellite DNA (Queller *et al.* 1993) which provide a source of highly polymorphic nuclear genes for the study of fine-scale population structure. Knowledge of population genetics is essential for conservation of natural populations and management of aquaculture broodstock.

Microsatellites have been the most widely used as marker of choice in various fields of genetic research. They become a powerful tool for assessing genetic diversity of wild and cultured stocks of aquatic species as well as monitoring of genetic change in selection program. This wide range of applications is due to their abundance, neutrality, co-dominant expression, high levels of polymorphism, and PCR-based analysis. Moreover, sample collection for microsatellite analysis which requires only a small pieces of fin clip preserved in alcohol, is an additional advantage of microsatellite over protein markers such as allozyme (Wright and Bentzen 1994).

Microsatellites are non-coding repetitive DNA regions comprise tandem repeated sequences of one to six nucleotides with the number of repeats ranges from 8 to 40 copies (Chistiakov *et al.* 2005). Most of microsatellites (30-70%) found in genome of vertebrates are di-nucleotide repeats such as $(AC)_n$, $(AT)_n$, and $(CG)_n$. Microsatellites with tri-, tetra-, and penta-nucleotide repeats are found at lower frequencies than the di-nucleotide repeats. Different number of repeat units results in microsatellite diversity among individuals in species or populations. The DNA replication slippage is thought to be the predominant mutation mechanism generating microsatellite variability. This mutation process occurs at the repetitive sequences when the new strand mis-pairs with the template strand, altering the repeat number of microsatellites (Schlötterer 2000).

Microsatellites have been isolated and characterized in a large number of fish species and have been used in a wide range of applications, as in evolutionary

biology, population genetics and ecology (Estoup and Angers 1998). Microsatellite loci are often found to be conserved among closely-related species. Cross-species amplifications of microsatellites have been demonstrated in various species of teleost fish but the percentage of loci that amplify successfully may decrease with increasing genetic distance, for example, sea bass *Dicentrarchus labrax* (Chistiakov *et al.* 2005), common carp *C. carpio* (Wang and Li 2007), Indian major carp *C. catla* (McConnell and Skibinaski 2000), Atlantic salmon *Salmon salar* (Skaala *et al.* 2004) Brazilian freshwater fish *Brycon opalinus* (Barroso *et al.* 2005), and *C. mrigala* (Lal *et al.* 2003).

The applications of microsatellites for assessing genetic variability in natural and cultured stocks have become common in genetic improvement program of aquaculture species. Lundrigan *et al.* (2004) compared the amount of genetic variation between natural populations and aquaculture strains of Arctic charr *Salvelinus alpinus*, using six microsatellite loci. Results indicated that hatchery strains and natural populations were genetically different and that small founding population sizes may have contributed to reduced genetic variation in hatchery strains. Li *et al.* (2003) assessed loss of genetic variation in hatchery strains and wild populations of Pacific abalone, *Haliotis discus hannai* using six microsatellite markers. All hatchery strains showed significant differentiation than wild populations. Results of this study suggested bottleneck effects when strains were founded in hatcheries. Alam and Islam (2005) used 12 microsatellite loci to investigate allelic variability and identification of individuals of Atlantic salmon *Salmo salar*. All hatchery strains exhibited 58% of the allelic richness than wild salmon.

5.1 *Genetic Diversity of C. cirrhosus*

A few works on genetic diversity of *C. cirrhosus* have been published. In their investigation, Lal *et al*. (2003) tested 44 microsatellite loci developed from four other cyprinids (*C. carpio*, *C. catla*, *Barbus barbus*, and *Barbodes gonionotus*) for

cross-species amplification with *C. cirrhosus* in which, successful amplifications were observed at 14 loci. Furthermore, five polymorphic loci (*MFW1*, *MFW2*, *MFW17*, *Barb45*, and *Bgon22*) were used to determine genetic variation among five riverine populations of *C. cirrhosus* from India. The authors reported relatively low genetic diversity within populations with the number of alleles ranging from 2 to 4 and the average observed heterozygosity ranging from 0.247 to 0.33 due to the small population size (76 specimens).

In the other study, Chauhan *et al.* (2007) examined genetic variation in wild populations of *C. cirrhosus* from ten rivers, belonging to four different river basins in India. Twenty-four enzymes and seven microsatellite loci developed from *C. carpio*, *B. barbus*, *B. gonionotus* and *Lebeo rohita*, were used to genotype a total of 680 samples. Analysis of microsatellite data revealed moderate genetic variation with allele diversity ranging from 2 to 7 and average observed heterozygosity ranging from 0.38 to 0.42. AMOVA analysis and the F_{st} value (0.013) revealed weak population structure of wild mrigal and suggested possible gene flow between rivers in different river basins.

MATERIALS AND METHODS

Materials

1. Incubator, Microcentrifuge, Vortex, Spectrophotometer, PCR machine, Polyachylamide gel sequencing machine, pH meter, Hot oven and Freezer.

2. Absolute ethanol, TNES-Urea, Proteinase-K, Phenol, Chloroform, Isoamyl alcohol, TE buffer, Agarose powder, TBE buffer, Ethidium bromide and PCR amplification

Methods

1. Sample collection

A total of 427 individuals of mrigal were collected from five hatcheries and five wild stocks during April to September, 2007 in Myanmar (Table 1). Five hatchery samples were obtained from breeders of 2-5 kg in five Fisheries Stations, located in different provinces under the Department of Fisheries (symbol: MBH, MSH, MPY, MHL, MTA) (Figure 3). Five wild samples were obtained from the traditional fishermen by trapping cast net in three locations of Ayeyarwaddy (symbol: MSHW, MPW, MHI) and two locations of Thanlwin river (symbol: MKA, MMO) (Figure 3). In addition, 43 broodstock samples from a hatchery in Thai Nguyen province, northern Vietnam were also collected for comparative purposes. Fin clip samples were preserved in 95% absolute ethanol until use.

Table 1 Original source, location, sample name, and number of hatchery and wild populations of *C. cirrhosus* in Myanmar and Vietnam

Sample Type	Sample Name	Location	Original Source	Number
Wild	MSHW	Shwebo	Ayeyarwaddy River	49
Wild	MPW	Pyintphue	Ayeyarwaddy River	49
Wild	MHI	Hinthada	Ayeyarwaddy River	50
Wild	MKA	Kayin	Thanlwin River	50
Wild	MMO	Mon	Thanlwin River	13
Hatchery	MBH	Kachin State Hatchery, Bhanmaw	Mandalay fisheries station	42
Hatchery	MSH	Sagaing Divi s ion Hatchery, Shwebo	Mandalay fisheries station	30
Hatchery	MPY	Mandalay Division Hatchery, Pyinmana	Mandalay fisheries station	50
Hatchery	MHL	Yangon Division Hatchery, Hlaw-gar	Tributaries of Ayeyarwaddy and leasable fisheries	50
Hatchery	MTA	Ayeyarwaddy Division Hatchery, Taloakla	Tributaries of Ayeyarwaddy River	44
Hatchery	VN	Private hatchery, Thai Nguyen Province, Vietnam		43

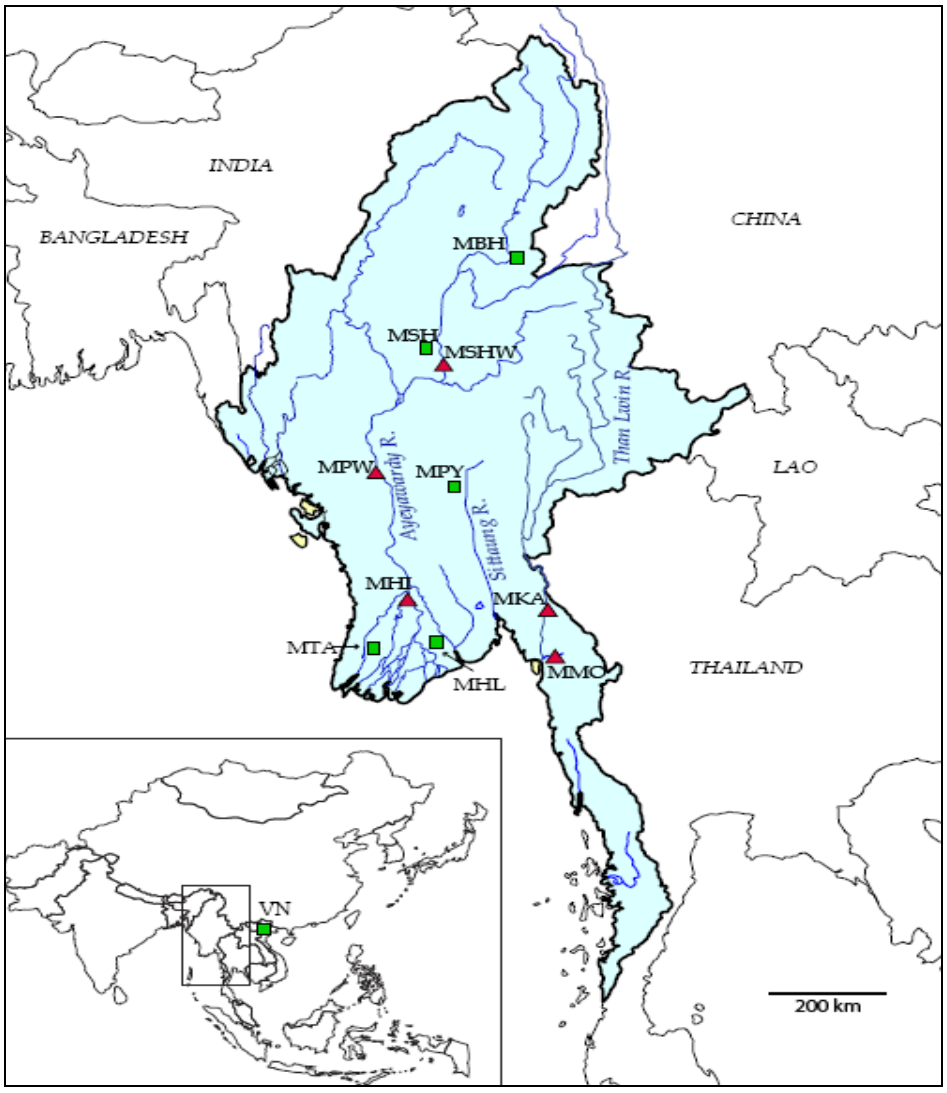

Figure 3 Map showing sampling sites of *C. cirrhosus* in this study

2. DNA Extraction

DNA extraction was carried out following standard procedures **(Taggart *et al.* 1992)** at Fish Genetics Laboratory, Department of Aquaculture, Faculty of Fisheries, Kasetsart University. For each sample, approximately 20-50 mg of fin tissues was cut

and air dried on the tissue papers and quickly placed in a microcentrifuge tubes. Four hundred µl TNES-Urea (10 mM Tris-HCl pH 7.5; 125 mM NaCl; 10 mM EDTA pH 7.5; 0.5% SDS; 4M Urea) extraction buffer and 4 µl of 20 mg/ml proteinase K were added to a 1.5ml microcentrifuge tube after few minute. The tube was inverted several times by hand and briefly vortexed. Then the tissue mixture was incubated at 37ºC in an incubator for 12-16 hr or overnight to break up cellular tissue and digest protein.

At the end of the incubation, 400 µl of phenol (pH 8.0 equilibrated) was added to the tube which was gently mixed until an emulsion was formed. The tubes were placed at room temperature for 10min, before centrifuged at 14,000 rpm for 10 min.

The viscous aqueous phase (the top layer) was transferred into a new tube using a wide pore pipette tip, and care was taken not to disturb the white interface which was formed by protein precipitation at this stage to move. Four hundred µl of phenol: chloroform: isoamyl alcohol (25:24:1) were then added to each tube. The mixture was mixed by inversion and the tubes were centrifuged again.

The top phase was transferred to a sterilised microcentrifuge tubes and 400 µl of chloroform: isoamyl alcohol (24: 1) were added to the tubes before centrifuge again. The solution was decanted and DNA was precipitated by adding 200 µl of cold absolute ethanol (95%) and the tube was swirled until the solution was thoroughly mixed, then centrifuged for 15 min at 14,000 rpm to collect the DNA pellet. The DNA pellet was washed in 100 µl of 70% ethanol.

After washing, the DNA pellet was allowed to air-dry by inverting the tubes on the tissue paper at room temperature for15-30 min. The dry DNA pellet was

resuspended in 150-200 µl of sterilised TE buffer (10mM Tris-HCL pH7.5; 1mM EDTA, pH 8.0) depending on the size of the pellet and placed at the room temperature for 24 hr. Finally, DNA was dissolved and a proportion of which was diluted to the concentration of 10ng/µl. Both stock and diluted DNA were stored at -20ºC until use. Two methods were used to determine the quantity and quality of the extracted DNA: 1) spectrophotometry and 2) agarose gel electrophoresis.

2.1 Spectrophotometry

The stock DNA solution was diluted to a 5/100 volumes, made up to 100 µl and quantified by measuring absorbance at 260 nm with a Gene Quant Pro® Spectrophotometer. Quantity of DNA was calculated as follows:

$$\text{DNA concentration (ng/ µl)} = (\text{Abs}_{260\ nm} \times 50) \times \text{dilution factor}$$

$\text{Abs}_{260\ nm}$ is an absorbance wavelength (260 nanometer), 50 is a concentration of DNA for each $\text{Abs}_{260\ nm}$ equal to one. DNA quality was assessed by determining the ratio of 260 nm by 280 nm. The ratio values between 1.8 and 2.0 indicate good DNA quality (Paul *et al*. 2001).

2.2 Agarose gel electrophoresis

Two µl of each stock sample solution were mixed with 1µl of loading dye in a well of 1% agarose gel. One µl of DNA marker standard (Gene Ruler 100bp DNA ladder) was also loaded in a separate well. The gel was run in TBE buffer (1M Tris-base) at 100 V for 30 min then stained in EtBr for 20 min and visualized under UV light or gel documentation system. DNA bands were observed on UV-

Transilluminator and photographed with a Gel Cam Polaroid camera. The quantity of DNA was measured by comparing with the intensity of the DNA maker standard, Gene Ruler 100bp DNA ladder.

3. Polymerase Chain Reaction

Seven microsatellite loci, *Bgon*22, *Lr*3, *Lr*12, *Lr*21, *Lr24*, *MFW*1 and *MFW*17, which were developed for other carp species were used. Details on the seven primers are given in Table 2.

Each forward primer was labeled with a fluorescent dye (Applied Biosystems, USA) (Table 2). Three multiplex PCR were conducted for each individual sample. Each PCR was conducted in a total volume of 6.25 µl, containing 10 ng of template DNA, 2x QIAGEN Multiplex PCR Master Mix (QiaGen Inc., USA) and 0.2 µM of each forward and reverse primer.

Loci included in each of the multiplex were as follows:

- Set 1: *Bgon22, Lr3, Lr12, and MFW1*
- Set 2: *Lr21 and Lr24*
- Set 3: *MFW17*.

Thermal cycles employed were: initial denature at 95°C for 15 min, followed by 29 cycles of 94°C for 30 s, 57°C (for Set 1 and 2) or 50°C (for Set 3) for 1 min 30 s and 72°C for 1 min, and then a final extension at 60°C for 30 min. Five µl of PCR products of Set 3 were added to appropriate samples of Set 2. The amplified products were sent to GeneSystems Co. Ltd (Bangkok, Thailand) for fragment analysis.

Table 2 Characteristics of microsatellite loci used in the present study

Locus	Primer sequences (5'-3')	Source	Repeat motif	Original species
Bgon 22	F-VIC-TCTTGTTGATCACACGGACG	Kamonrat *et al.* 2002	CCT	*B.gonionotus*
	R-ACAGATGGGGAAAGAGAGCA			
*Lr*3	F-NED-ATCTGGCTGCCTATTCACC	Das *et al.* 2004	TG	*L. rohita*
	R-CATCGGCGACTGCACTGGA			
*Lr*12	F-VIC-CACCGCTGCTGTCCATCA	Das *et al.* 2004	CA	*L. rohita*
	R-AGGTCGGCCAGATACACG			
MFW1	F-NED-GTCCAGACTGTTCATCAGGAG	Croojimans *et al.* 1997	CA	*C. carpio*
	R-GAGGTGTACACTGAGTCACGC			
*Lr*21	F-VIC- GATCAGAGGGTCAATGTGG	Das *et al.* 2004	CA	*L. rohita*
	R-CAGCAGAGTACTATGGAAGA			
Lr24	F-NED-CAAGGCCAAAAGTGTCCAT	Das *et al.* 2004	TG	*L. rohita*
	R-AGGAAATTGGTAAAGTGTTTC			
*MFW*17	F-VIC-CAACTACAGAGAAATTTCATC	Croojimans *et al.* 1997	CA	*C. carpio*
	R-GAAATGGTACATGACCTCAAG			

4. Statistical analysis

Microsatellite alleles were scored using GeneMapper 4.0 (Applied Biosystem). Data were exported to an Excel worksheet and further exported into format required by different software packages using Microsatellite Tool Kit (Park *et al* 2001). Micro-checker v 2.2.3 (Oosternhout *et al*., 2004) was used to identify the presence of genotyping error due to the presence of null allele, stuttering or large allele dropt out.

4.1 Genetic variation within populations

Number of alleles at each locus of each sample and allele richness was estimated using FSTAT version 2.9.3 (Goudet 2001). ARLEQUIN 3.11 (Schneider *et al.* 2000) was used to estimate observed and expected heterozygosities. CONVERT version 1.31 (Glaubitz 2004) was used to calculate allele frequencies and identifying private alleles. GENEPOP 4.0 (Raymond and Rousset 1995; Rousset 2008) was used to test genotypic distributions for conformance to Hardy-Weinberg expectations, and to test the loci for linkage disequilibrium.

4.2 Genetic diversity between populations

Levels of genetic differentiation were assessed using the estimates of pairwise F_{ST} as implemented in ARLEQUIN 3.11 (Schneider *et al.* 2000). Levels of significance of these values were calculated using the same software. Homogeneity of microsatellite allele and genotype frequencies between samples was assessed by exact tests using Genepop 4.0, with results combined across loci using Fisher's method. The sequential Bonferroni adjustments (Rice 1989) were applied to correct

for the effect of multiple tests. Genetic relationships between samples were visualised by multidimensional scaling (MDS) of F_{ST} values using SAS version 9.0 (SAS Institute Inc., USA).

Analysis of molecular variance (AMOVA) incorporated in ARLEQUIN 3.11 (Excoffier *et al.* 1992) was used to partition genetic variance hierarchically between (1) wild and hatchery samples from Myanmar and (2) samples from Myanmar and the hatchery sample from Vietnam (VN) and (3) groups identified by MDS analysis.

Assignment tests were performed using the program STRUCTURE version 2.2 (Pritchard *et al.* 2000). Due to the small number of loci and often STRUCTURE could detect only deep division amongst samples, the value of $k = 5$ was used as identified in AMOVA. Five runs were performed using a burn-in time of 300,000 followed by 200,000 iterations.

RESULTS

1. Microsatellite amplification and allele scoring

All seven loci were successfully amplified in mrigal samples. One locus (*Lr24*) experienced split peaks due to incomplete adenylation of PCR products. Examples of microsatellite electropherogram are shown in Figure 4. Allele frequency data for each population were presented in Appendix Table 1.

Null alleles were found at loci *Bgon22* and *Lr3* for MKA, at *Bgon22* for MMO in the wild populations, at *MFW17* for MBH, at *Lr3* for MSH in the hatchery populations and at *MFW1* in hatchery samples from Vietnam. Because the presence of null alleles at locus *Lr24* was detected in all samples, the locus was not included in further analysis.

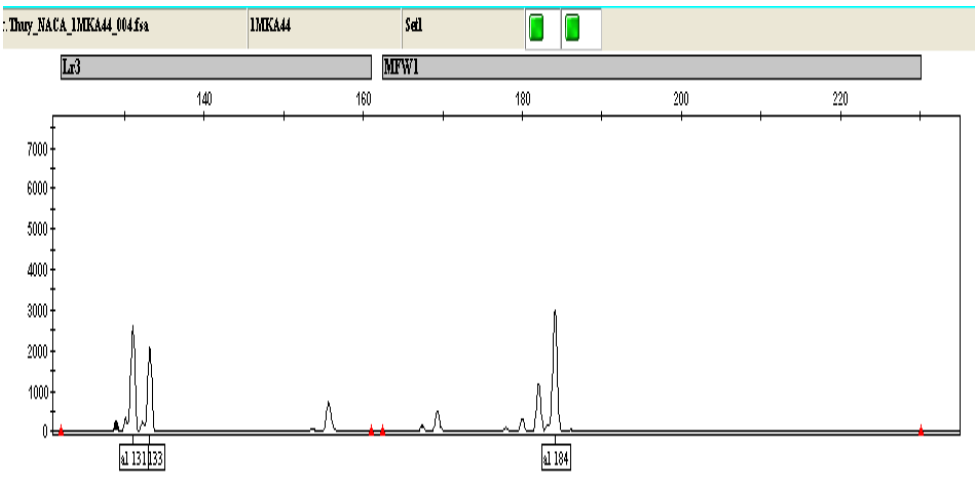

Figure 4 Microsatellite electropherogram of all loci

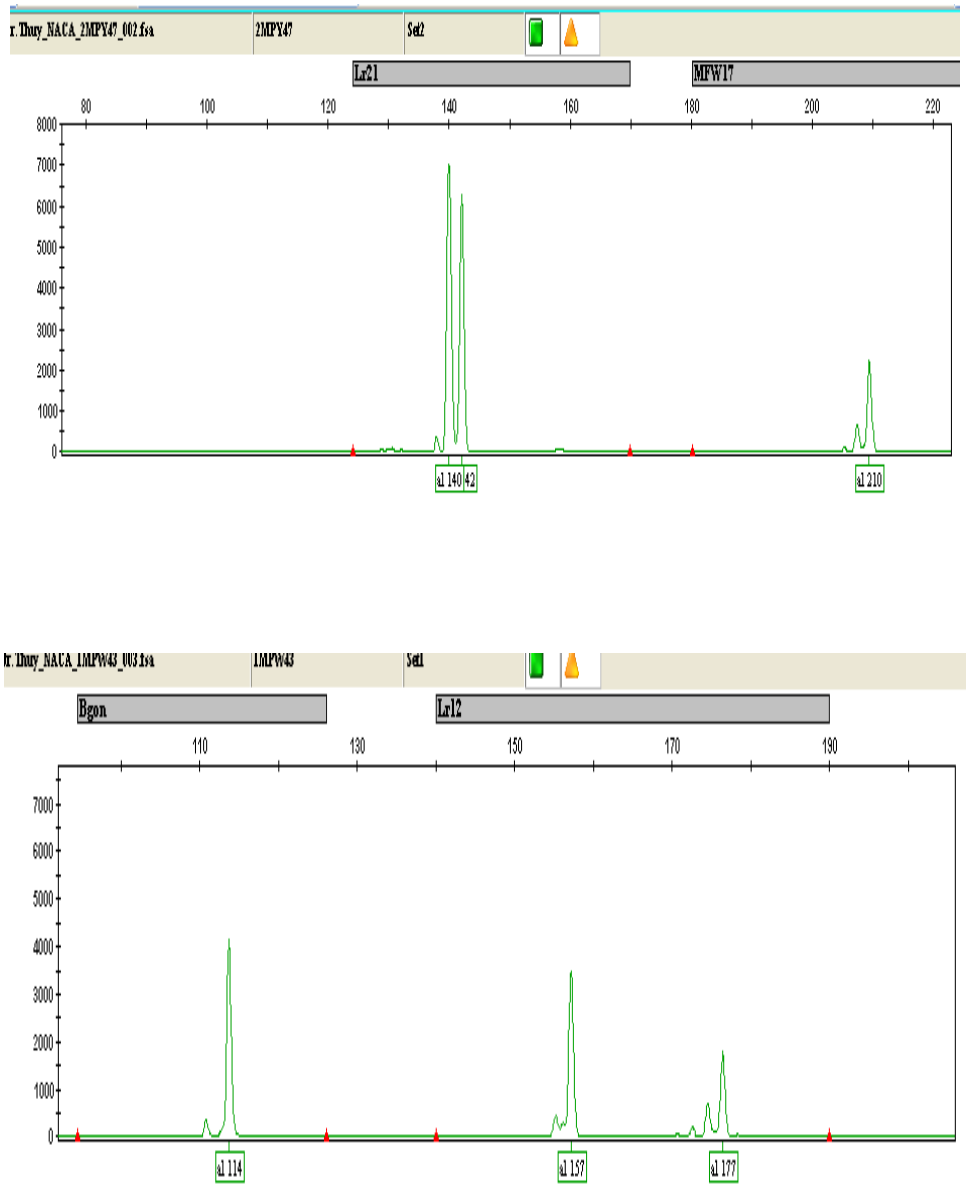

Figure 4 (Continued)

2. Within population variation

2.1 Allele frequency, number of alleles per locus, and allele richness

Six microsatellite loci were highly polymorphic in all populations of Myanmar, showing different degree of variability across loci but two loci were monomorphic in Vietnam populations. A total of 112 alleles were detected at six loci in 470 individuals, ranging from 10 alleles at locus *Bgon22* to 29 alleles at locus *MFW17*. Loci *MFW1*, *Lr3, Lr12,* and *Lr21* were polymorphic with 27, 17, 17, and 12 alleles respectively.

For Myanmar populations, mean number of allelic richness (A_r) in the hatchery samples ranged from 5.24 (MPY) to 8.41 (MTA), and in the wild samples ranged from 5.34 (MSHW) to 8.52 (MKA). Vietnam hatchery stock, however, exhibited lower value of allelic richness at 2.31 (Table 3).

Mean number of alleles per locus (A_n) detected in the hatchery populations ranged from 7.2 to 13.2 and in the wild populations ranged from 7.2 to 13.0, and 2.5 in the hatchery samples of Vietnam.

Private alleles were found at all loci in Myanmar populations, but at one locus in Vietnam hatchery population. More specifically, private alleles were observed in two hatchery and four wild populations at all loci, i.e., *Bgon22* (102), *Lr3* (145, 147, 151, 155, and 161), *Lr12* (183), *Lr21* (164), *MFW1* (158 and 160) and *MFW17* (190 and 248) in Myanmar samples and, at *MFW17* (116) for Vietnam samples. The frequencies of common alleles at the six microsatellite loci ranged from 0.39 to 0.96.

Fixation of alleles was observed in Vietnam samples at loci *Lr3* (135) and *Lr21* (140) and was absent in other populations. Allele number, allele frequencies, private allele and fixation allele at the six microsatellite loci in the populations from Myanmar and Vietnam are shown in Appendix table 1.

2.2 Observed and expected heterozygosities

Observed heterozygosities across loci ranged from 0.55 for MSHW to 0.74 for MPW in wild samples and from 0.654 for MPY to 0.739 for MTA in the Myanmar hatchery populations. Expected heterozygosities ranged from 0.56 for MSHW to 0.79 for MKA in the wild samples and from 0.69 for MPY to 0.0.79 for MSH in the Myanmar hatchery populations. Observed and expected heterozygosities of Vietnam samples were 0.40 and 0.52. Heterozygote deficiency was observed at locus *Lr3* in two samples (MPW and MSH), at locus *MFW1* in VN and at three loci (*Bgon22*, *Lr21*, and *MFW17*) in MKA (Table 3).

Table 3 Genetic variation at six microsatellite loci in eleven populations including sample size (N), total number of alleles (A), allelic richness (A_r), observed heterozygosity (H_o), expected heterozygosity (H_e), fixation index (F_{IS}), and P-value for test of HW expectations (HWE)

Population		MSHW	MPW	MHI	MKA	MMO	MBH	MSH	MTA	MHL	MPY	VN
Sample size (N)		49	49	50	50	13	42	30	44	50	50	47
Locus												
Bgon22	*A*	6	6	7	9	6	8	6	7	8	3	3
	A_r	3.46	4.92	5.41	6.63	6.00	5.95	3.46	5.02	5.68	3.00	2.60
	H_o	0.551	0.571	0.580	0.520	0.538	0.619	0.600	0.614	0.700	0.520	0.458
	H_e	0.529	0.668	0.683	0.746	0.794	0.713	0.712	0.700	0.731	0.594	0.434
	F_{IS}	-0.043	0.145	0.153	0.305	0.331	0.133	0.160	0.125	0.043	0.125	-0.057
	HWE	1.000	0.004	0.017	0.000	0.018	0.456	0.000	0.355	0.167	0.521	0.873
Lr3	*A*	6	12	11	7	4	7	7	7	9	6	1
	A_r	3.50	7.07	6.36	5.70	4.00	5.30	5.69	5.20	5.10	3.38	1.00
	H_o	0.265	0.592	0.620	0.540	0.462	0.548	0.400	0.500	0.580	0.360	-
	H_e	0.293	0.667	0.662	0.666	0.502	0.535	0.702	0.517	0.579	0.404	-
	F_{IS}	0.095	0.113	0.064	0.191	0.083	-0.024	0.435	0.034	-0.002	0.110	-
	HWE	0.076	0.258	0.389	0.097	0.297	0.654	0.000	0.669	0.331	0.680	-
Lr12		10	14	16	15	11	14	11	15	12	6	3
	A_r	7.50	10.15	9.89	10.76	11.00	10.16	9.12	10.68	7.75	5.42	2.99
	H_o	0.857	0.857	0.860	0.920	0.923	0.857	0.833	0.909	0.860	0.720	0.469
	H_e	0.832	0.894	0.880	0.904	0.920	0.893	0.847	0.902	0.832	0.801	0.435
	F_{IS}	-0.030	0.041	0.023	-0.018	-0.004	0.040	0.016	-0.008	-0.035	0.102	-0.079
	HWE	0.000	0.066	0.221	0.192	0.294	0.409	0.054	0.500	0.189	0.550	0.839
Lr21	*A*	2	7	6	10	4	9	6	6	6	5	1
	A_r	1.70	4.60	3.76	6.44	4.00	5.25	4.94	4.40	4.34	3.70	1.00
	H_o	0.082	0.571	0.480	0.580	0.615	0.667	0.667	0.659	0.460	0.640	-
	H_e	0.079	0.622	0.489	0.675	0.582	0.605	0.675	0.539	0.532	0.551	-
	F_{IS}	-0.032	0.082	0.019	0.142	-0.061	-0.103	0.013	-0.226	0.137	-0.164	-
	HWE	1.000	0.010	0.382	0.001	0.628	0.126	0.128	0.736	0.059	0.559	-

Table 3 (Continued)

Population		MSHW	MPW	MHI	MKA	MMO	MBH	MSH	MTA	MHL	MPY	VN
Locus												
MFW1	*A*	10	16	18	15	8	18	11	21	18	13	2
	A_r	7.12	10.18	11.33	9.45	8.00	10.50	9.18	11.89	10.61	8.35	2.00
	H_o	0.694	0.918	0.920	0.820	0.769	0.810	0.800	0.886	0.840	0.820	0.111
	H_e	0.780	0.883	0.887	0.853	0.883	0.874	0.881	0.905	0.874	0.855	0.505
	F_{IS}	0.112	-0.040	-0.037	0.039	0.134	0.074	0.093	0.021	0.039	0.041	0.782
	HWE	0.000	0.096	0.199	0.643	0.028	0.235	0.022	0.274	0.000	0.303	0.000
MFW 17	*A*	14	17	20	21	10	19	13	23	17	10	5
	A_r	8.70	11.34	11.75	12.16	10.00	12.86	10.31	13.24	10.81	7.65	4.34
	H_o	0.872	0.898	0.820	0.809	0.846	0.825	0.933	0.864	0.880	0.860	0.563
	H_e	0.834	0.895	0.896	0.900	0.892	0.925	0.898	0.920	0.892	0.813	0.695
	F_{IS}	-0.047	-0.004	0.085	0.102	0.054	0.109	-0.040	0.062	0.014	-0.059	0.192
	HWE	0.000	0.059	0.267	0.081	0.298	0.001	0.001	0.440	0.000	0.193	0.000
Average across loci												
	A	8.00±4.20	12.0±4.60	12.9±5.86	13.0±5.15	7.17±3.00	12.5±5.24	9.0±3.03	13.2±7.60	11.7±4.93	7.17±3.66	2.17±1.52
	A_r	5.34±2.80	8.04±2.91	8.09±3.35	8.52±2.65	7.17±3.00	8.34±3.25	7.46±2.78	8.41±3.96	7.38±2.82	5.24±2.30	2.31±1.28
	H_o	0.55±0.10	0.74±0.02	0.71±0.03	0.69±0.03	0.69±0.03	0.72±0.01	0.71±0.04	0.74±0.03	0.72±0.03	0.65±0.04	0.40±0.07
	H_e	0.56±0.10	0.77±0.01	0.75±0.03	0.79±0.01	0.76±0.03	0.76±0.03	0.79±0.01	0.75±0.04	0.74±0.02	0.69±0.03	0.52±0.08
	F_{IS}	-0.002	0.048	0.049	0.108	0.095	0.039	0.104	0.012	0.027	0.024	0.193
	HWE	0.00556	0.00833	0.01667	0.00500	0.01250	0.01000	0.00714	0.05000	0.00455	0.02500	0.00625

Note: for single locus HWE, *P*-value = 0.0045 after sequential Bonferroni correction for all loci HWE, *P*-value = 0.0083 after sequential Bonferroni Correction.

2.3 Hardy-Weinberg equilibrium and linkage disequilibrium

Genotypic frequencies of eleven populations at six loci were tested for conformation to HWE using a probability test. Six populations (MSHW, MPW, MKA, MSH, MHL, VN) showed significant deviations from H-W equilibrium for all loci after sequential Bonferroni adjustment. Single-locus exact tests for HWE indicated significant deviations in 11 out of 64 tests in two hatchery and two wild populations from Myanmar and a hatchery sample of Vietnam after sequential Bonferroni correction (Table 3).

Pairwise linkage (genotypic) disequilibrium in each population was tested using a Markov chain method. The probability test revealed that linkage disequilibrium was evidenced in 4 out of 165 available tests for eleven populations after sequential Bonferroni correction.

3. Genetic differentiation between populations

3.1 Pairwise F_{ST}

Overall and single locus estimates of F_{ST} values indicated high levels of differentiation among populations. The mean F_{ST} value across all loci was 0.134 ($CI_{95\%}$ = 0.079-0.180). The F_{ST} values (0 to 1) display the levels of genetic differentiation between populations. The values of 0 to 0.05, 0.05 to 0.15, and 0.15 to 0.25, respectively indicate low, moderate, and high level of population differentiation (Wright 1978).

Pairwise F_{ST} estimates and level of significance for all population pairs are presented in Table 5. F_{ST} among Myanmar samples ranged from -0.005 (between MMO and MTA) to 0.095 (between MSH and MSHW), and that among Myanmar with VN sample were from 0.375 to 0.537. Of 55 pairwise comparisons, eleven F_{ST} values were significant. Four samples MSHW, MHL, MPY and VN showed significant F_{ST} with all other samples. Results of exact tests for population differentiation were in accordance with pairwise F_{ST} estimates, and additionally detected significant differentiations in two additional pairwise tests (Table 5).

Table 4 Weir & Cockerham estimate of F_{ST} among samples, among individuals within samples and within individuals

Locus	F_{ST}
Bgon22	0.121
Lr3	0.229
Lr12	0.089
Lr 21	0.231
MFW 1	0.086
MFW17	0.050
All Loci	0.134
95% CI	0.079-0.180

Table 5 Matrix of pairwise F_{ST} an eleven populations

populations	MSHW	MPW	MHI	MKA	MMO	MBH	MSH	MTA	MHL	MPY	VN
MSHW	-	0.071	0.058	0.077	0.086	0.061	**0.095**	0.060	0.081	0.090	**0.537**
MPW		-	0.000	0.001	0.004	0.000	0.009	<0.001	0.013	0.034	0.395
MHI			-	0.005	0.003	0.000	0.011	0.002	0.012	0.043	0.408
MKA				-	0.006	0.006	0.011	0.003	0.024	0.039	**0.375**
MMO					-	0.001	0.012	<0.005	0.018	0.057	0.477
MBH						-	0.011	**<0.001**	0.006	0.038	0.426
MSH							-	0.016	0.029	0.061	0.388
MTA								-	0.013	0.033	0.419
MHL									-	0.045	0.436
MPY										-	0.456
VN											-

Note: Significant differentiation show in bold $P<0.05$

3.2 Multidimensional Scaling (MDS)

A multidimensional (MDS) plot based on pairwise F_{ST} values displayed genetic relationship among five groups of mrigal populations. The MDS plots clearly show the distinction of VN sample to the rest. With regard to samples from Myanmar, four groups were observed: (1) MSHW, (2) MPY, (3) MHL and (4) MPW, MMO, MKA, MSH, MBH, MHI and MTA (Figure 5).

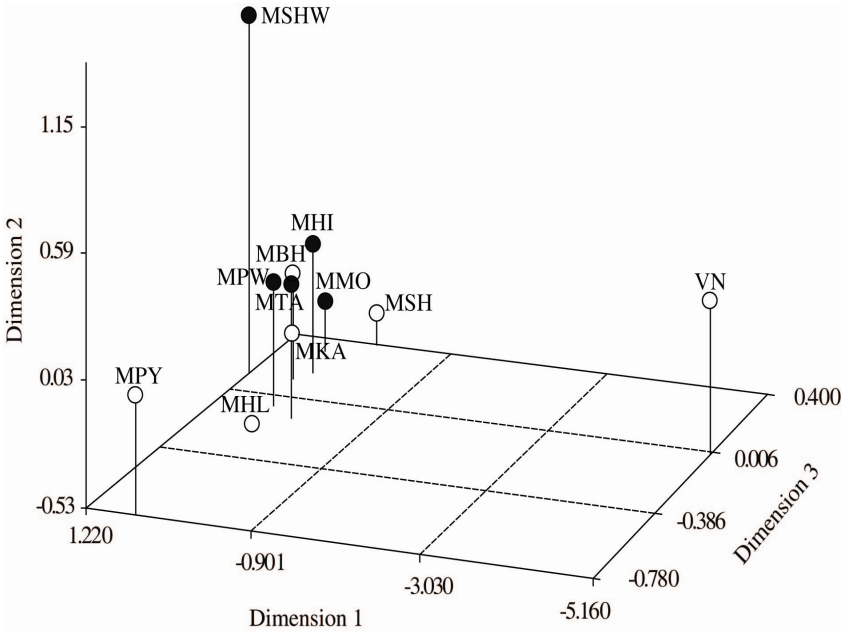

Figure 5 Multidimensional scale analysis (MDS) of pairwise F_{ST} values (stress = 0.045). Closed black circles are wild samples and white circles are hatchery samples

3.3 AMOVA

When wild and hatchery samples from Myanmar were pooled into two different groups, results of AMOVA analysis indicates that there was no genetic

differentiation between these groups (F_{CT} = 0.000; P>0.05), and 97.15% of genetic variation could be attributed to within populations variation (F_{ST} = 0.028; P<0.05) (Table 8). In contrast, when all Myanmar samples were pooled and VN sample served as another group, significant differentiation was observed among groups (F_{CT} = 0.345; P<0.05), and variation among groups and within populations contributed significantly (34.51% and 63.47%, respectively) to overall genetic variation attributed to among groups. Similarly, when grouping was based on the results of MDS analysis, variation among groups also contributed significantly (16.29%) to overall variation. In all cases, contributions of variation among populations within groups were relatively small (0.94-2.86%) (Table 6).

Table 6 Percentage variation and fixation indices inferred from AMOVA analyses with 10000 permutations; (1) between wild and hatchery samples from Myanmar, (2) between Vietnam and all Myanmar samples, and (3) grouping based on MDS analysis results

Source of variation	(1)		(2)		(3)	
	Percentage variation	Fixation indices	Percentage variation	Fixation indices	Percentage variation	Fixation indices
Among groups	-0.04	0.000	34.51	0.345*	16.29	0.163*
Among populations within groups	2.86	0.029*	2.01	0.031*	0.94	0.011*
Within populations	97.17	0.028*	63.47	0.365*	82.77	0.172*

Note: Asterisk (*) indicates the value was significant at P<0.05

3.4 Cluster Analysis

Results of the Bayesian clustering analysis at $k = 5$ are given in Table 7 and Figure 6, and overall in accordance to that of the MDS analysis. All individuals of the VN sample were cleanly assigned to one cluster with the mean proportion of memberships ranged of 0.983. MSHW and MPY individuals were grouped into two other clusters with the mean proportion of memberships of 0.756 and 0.828, respectively. Sample MHL appeared more admixed; however, a large number of individuals (19) of this sample formed another cluster with proportion of membership over 0.800.

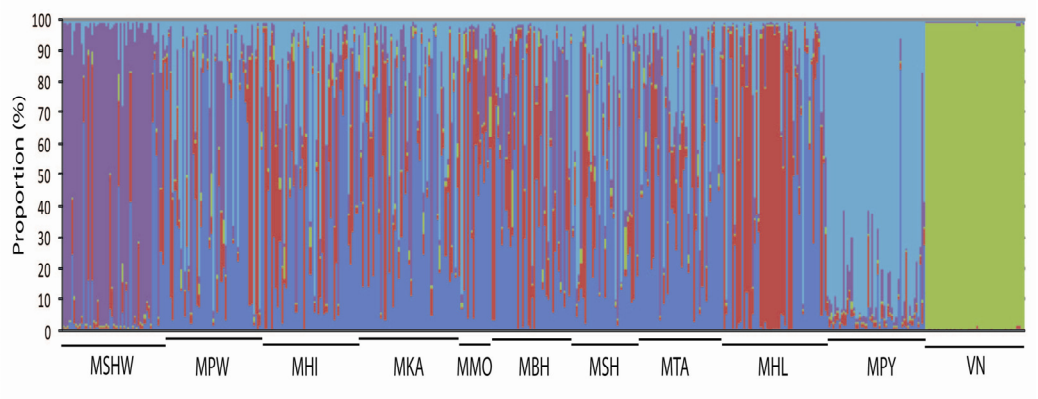

Figure 6 Proportional membership (Q) of each individual of mrigal in the five clusters identified by STRUCTURE. The locality of origin for each individual is indicated in the X-axis

Table 7 Proportion of membership of each mrigal sample in each of the five genetic clusters identified by STRUCTURE. Bolding highlights values of proportional membership > 0.75 to a single genetic cluster for a locality

Sample	Cluster				
	1	2	3	4	5
MSHW	**0.756**	0.039	0.089	0.109	0.008
MPW	0.133	0.258	0.186	0.410	0.013
MHI	0.128	0.192	0.257	0.407	0.016
MKA	0.141	0.211	0.162	0.464	0.022
MMO	0.170	0.059	0.292	0.463	0.015
MBH	0.174	0.153	0.285	0.371	0.017
MSH	0.178	0.300	0.177	0.323	0.023
MTA	0.143	0.203	0.162	0.481	0.011
MHL	0.070	0.140	0.484	0.299	0.007
MPY	0.073	**0.828**	0.031	0.063	0.005
VN	0.004	0.004	0.004	0.004	**0.983**

DISCUSSION

Within population genetic diversity

The levels of genetic diversity within populations from Myanmar were high at all loci as evident from high number of allele richness (A_r = 5.24-8.52), high average number of allele (A = 7.2-13.2) and high levels of expected heterozygosity (H_e = 0.558-0.791). The high amount of microsatellite variation may indicate large effective sizes of the studied populations because populations with large effective sizes tend to retain high numbers of alleles originating from large number of founder populations (Norris *et al.* 1999; Yamashita *et al.* 2005). The Vietnam samples, however, displayed lower levels of genetic variation than the Myanmar samples with the average allele richness of 2.31 and the expected heterozygosity of 0.517. The small number of alleles and low levels of expected heterozygosity are usually caused by the limited number of captive and founder stock in the hatchery and/or inbreeding events when the population was founded (Li *et al.* 2007).

A total of 112 alleles were detected at the six loci in 470 individuals of eleven populations. Number of alleles for *Bgon22*, *Lr3*, *Lr12*, *Lr21*, *MFW1* and *MFW17* were 10, 17, 17, 12, 27 and 29 respectively. Loci *MFW1* and *MFW17* were highly polymorphic as compared with a study by Lal *et al.* (2003) in five wild populations from India, of which 5 and 16 alleles were observed for *MFW1* and *MFW17*. In addition, expected heterozygosity reported presently was higher than that of previous investigation by (Chauhan *et al.* 2007) (H_e = 0.380 – 0.402).

Private alleles were detected in three wild populations (MPW, MHI and MKA) and three hatchery stocks (MBH, MTA, and MPY) of Myanmar samples. Among them, five private alleles were observed in MTA population. The presence of private alleles could have resulted from multiple population bottlenecks or single founding populations from an ancestral population with high allelic diversity (Schrodeder *et al.* 2009).

Fixation of allele was found only in Vietnam samples. In hatchery populations, fixation of alleles could have resulted from a small number of broodstock. At neutral loci, bottlenecks effect may theoretically have caused strong reductions in allelic richness, and a more limited decrease in gene diversity, since rare alleles are more readily affected by genetic drift than frequent alleles (Nei 1978). Genetic drift, like inbreeding, is inversely related to effective population size and is often a major cause of reduced variation in hatchery populations, especially where rare alleles are concerned (Tave, 1986). Theoretical and empirical studies involving fish show that small founding populations quickly lose most of rare alleles (Vrijenhoek 1998).

In the present study, heterozygote deficits were detected in two wild and two hatchery populations. Null or non-amplified alleles were likely to be the cause for the deviations from Hardy-Weinberg expectation. Null alleles may affect the estimation of population differentiation by reducing the genetic diversity within populations (Chow *et al.* 1988; Chauhan *et al.* 2007). Heterozygote deficiency normally occurs in mixed populations as a result of Wahlund effect. Heterozygosity deficit can occur not only after a recent change of the effective population size but also if heterozygotes have a selective advantage or disadvantage (Cornuet and Luikart 1996).

Among the Myanmar samples, MSHW showed the lowest levels of genetic diversity than other populations in Myanmar. This is surprising as often hatchery samples show lower levels of genetic variation due to founder effects and genetic drift. This is not the case in Myanmar because hatcheries frequently recruit new genetic materials from a number of fisheries and also release fingerlings into the rivers as part of the commitment to obtain a license. The releases often are made into the lower Ayeyarwaddy and its flood plains. MSHW is located at Shwebo, where stock enhancement is not practised and as such relatively isolated compared to others.

Sample VN appeared to be the least diverse among all, and possibly due to two reasons. First, mrigal was introduced into Vietnam in 1984 from Lao PDR, of stocks of Indian origin (Ministry of Fisheries 1996) which is less diverse compared to Myanmar samples. Unfortunately direct comparisons in terms of allele presence is not warranted as original wild counterparts of the VN sample were not reported, and the genotyping methods used are different in the two studies referred to previously. Secondly, mrigal was introduced into Vietnam once and there was no further recruitment of new genetic materials. The species is highly fecund (100,000 -154,000 fry per 1 kg of female broodstock) (Ministry of Fisheries 1996), and the tendency of keeping a small number of broodstock to reduce the cost of production, coupled with mass spawning practices by many hatcheries would have significantly resulted in reduction in genetic diversity in hatchery stocks.

Between population genetic differentiation

The results of the microsatellite analyses revealed a high degree of population substructure among mrigal samples with the overall F_{ST} value of 0.134. In all tests, deep division was observed between Myanmar and VN samples and significant structure was observed among Myanmar samples.

The division between Myanmar and Indian samples is likely to be related to isolation due to the independence of drainages (the Brahmaputtra-Ganges and the Ayeyarwaddy). The large accepted view is that the family Cyprinidae originated in southeastern Asia (Briggs 1979) and soon after India joined mainland Eurasia in Eocene (~57-35 mya) the westward migration of Malayan freshwater fauna began (Ali & Aitchison 2008). Subsequently, a number of river recapture events resulted in large-scale changes in drainage systems in the region (Brookfield 1998; Zeitler *et al.* 2001; Clark *et al.* 2004) and the Brahmaputta-Ganges drainages are believed to have

effectively separated from the Ayeyarwaddy by the Indoburman range, an orogen related to the collision of the Indian plate with the west Burma Block (Bannert and Helmcke 1981; Bender 1983). It is plausible that the uplift of the Indoburman ranges could have played an important role in shaping the current population structure of mrigal. Although no comparable information is available at species level, there is evidence that the Indoburman ranges have a role in evolution and distribution of the freshwater fish genera such as *Badis* (Rüber *et al.* 2004).

In freshwater populations, gene flow is only possible between rivers belonging to the same water system. VN sample with Indian origin were highly significance different ranged from (0.372- 0.532) compared to Myanmar samples. The Vietnam samples originated from India showed the lowest level of genetic diversity. Mrigal was introduced into Vietnam for over 20 years and no new genetic material has been procured. The stock has been maintained at the research institute for aquaculture no1 for breeding and seed were disseminated to a number of hatcheries which often use a small number of broodstock. As such this low level of genetic diversity in hatcheries in Vietnam is not surprising. McCauley *et al.* (1995) showed that F_{ST} can be increased by kin- structured colonization; that is, when relatives rather than random individuals are drawn from the same source population. Obligate freshwater fish are expected to display greater levels of genetic differentiation and population subdivision than marine species due to the isolating nature of river systems and small effective population sizes (McGlashan and Hughes 2000).

Within the Myanmar samples population genetic structure also exists. Four groups were identified including (1) MSHW, (2) MPY, (3) MHL and (4) the rest of the samples in Myanmar and Vietnam samples. Wild population of MPY, MSHW and MHL were alone placed in one cluster and the remaining the hatchery and wild samples were placed in other clusters. The VN stock clearly separated from the Myanmar populations. These samples are hatchery broodstock and it is important to identify their wild counterpart. The MSHW sample was from the upper section of the

Ayeyarwaddy and is genetically highly distinct to other samples. Stock enhancement mainly occurs in the lower reaches of the Ayeyarwaddy and in addition hatcheries only recruit broodstock from a number of fisheries in the lower Ayeyarwaddy basin but not in Shwebo, the locality of MSHW. As such MSHW is much isolated and as a result genetically differentiated to others.

Furthermore, anthropogenic influences such as channelisation and overfishing, as well as historical and physical factors such as barriers, droughts, salinity, siltation, pH, temperature, food supply, predators and disease, are all factors that may fragment gene pools and/or be of adaptive significance, all of which may enhance the possibility of population subdivision (Carvalho 1993).

It is interesting that the two hatchery samples MHL and MPY are highly differentiated from others. MHL was established over 50 years, 1958 without proper management of breeding records for domesticated stocks in hatcheries that is no record size and number of founder populations. Records are not available on the exact origins of broodstock from these two hatcheries and anecdotal evidence suggests that MHL recruits broodstock from the lower reaches of the Ayeyarwaddy in Yangon, while MPY does so from a number of fisheries in the Mandalay State. The latter is a floodplain with a number of lakes and floodplain depressions that could serve as a separate population unit and facilitate genetic changes due to isolation. The cryptic genetic structure observed herein warrants further investigation with a better genetic inventory with samples from a wider geographical range to gain a better understanding of population structure of mrigal in Myanmar. Although some areas are difficult to access, it will be beneficial to make attempts to obtain additional samples from other areas of their range of distribution.

In contrast to MSHW, MHL and MPY the rest of the samples appeared to be admixed and homogeneous and it could be brought about by stock enhancement and

translocation. Wild caught samples from the Thanlwin river system (MMO and MKA) are likely introduced stocks from the Ayeyarwaddy basin. Stock enhancement has been practiced in Myanmar since 1950s and hatcheries provide seeds for stocking as part of licensing protocols. Hatcheries in turn procure broodstock from managed leasable fisheries, often referred to as “Inn fisheries” in Myanmar (De Silva and Funge-Smith 2005). These fisheries use either non-perennial or perennial water bodies in the floodplains of the Ayeyarwaddy when at the onset of floods areas are fenced and leased by the government for fisheries purposes. The lessees could enhance by stocking the leased areas or depend entirely on the naturally recruited stocks.

CONCLUSIONS AND RECOMMENDATIONS

The present study provides information of genetic status of mrigal, an important aquaculture species in Myanmar as it is emerged as an export commodity. Assessing genetic diversity is one of the most important steps in managing fisheries resources and selective breeding programs for genetic improvement. Because aquaculture and enhancement programs of mrigal in the country are mainly dependent upon the studied populations, proper management strategies are required for the long-term exploitation of these resources. In addition, the current practices of releasing the fingerlings back to the floodplains need special attentions from the policy makers.

Overall, microsatellite analysis revealed high levels of genetic diversity in all ten populations of mrigal as well as a strong population substructure. Mrigal populations in Myanmar appear to possess great potential to changing environment compared to the Indian mrigal. Despite a long history of using unmanaged broodstocks, most wild and hatchery stocks still exhibited high amount of genetic diversity. Inbreeding was not detected in the studied populations. Although heterozygote deficits were detected at three loci in the wild population of MKA, inbreeding was unlikely the cause.

According to the multidimensional scaling (MDS) plot, four groups of genetically different populations of wild and hatchery samples in Myanmar were displayed: (1) MSHW, (2) MPY, (3) MHL and (4) MPW, MMO, MKA, MSH, MBH, MHI and MTA. The wild population of MSHW, and the hatchery populations of MPY and MHL should be managed separately from other groups and fingerlings from the hatcheries investigated herein should not be used to stock at MSHW location. Further investigation on structure of the wild stocks with sampling areas covering most of the mrigal fisheries in Myanmar is warranted. The remaining seven populations including wild and hatchery samples displayed mixed genetic composition and were grouped together in the cluster analysis.

Population dynamics of fisheries stock is highly dependent upon exploitation of wild stocks. Therefore, the necessary tools are needed for maintaining of these stocks. It is advisable that the authorities utilize the progeny of broodstock from the same locality for restocking purposes to maintain genetic integrity of wild populations. The results obtained from this study can be used to set the basic guidelines for management of wild populations and successful selective breeding programs of *C. cirrhinus* in Myanmar as well as to understand the risk of reducing genetic diversity in these populations.

Management considerations

Of five mrigal populations of wild origin, three were from the Ayeyarwaddy River including MSHW, MPW, and MHI whereas MKA and MMO were from the Thanlwin River. For hatchery stocks, three (MBH, MSH, and MPY) were originated from Mandalay fisheries station and two (MHL and MTA) were originated from the tributaries of the Ayeyarwaddy River and leasable fisheries. Among the wild populations of the Ayeyarwaddy River, MSHW was genetically isolated from the other two populations, possibly due to large geographical distance. The MPW and MHI were more similar and were grouped together in cluster analysis. The MSHW samples were collected from the Division of Sagaing, approximately 800.km and 2000km from Pwintphue at Magway Division and Hinthada samples at the Ayeyarwaddy Division, respectively. Further, MSHW had the lowest levels of genetic diversity as displayed by allelic richness and the values of heterozygosities. In addition, restocking programs have been undertaken regularly for MPW and MHI populations using fingerlings from the hatchery stations, but not for MSHW population. To preserve genetic characteristics of the MSHW population, management plan should be specifically designed. For instance, if restocking program is needed in the Shwebo area, broodstock should be collected from the local population to avoid genetic contamination and to benefit from local adaptation. Translocation of broodstock across the river systems should be avoided to prevent

genetic contamination and possible adversary effects of hybridization. Close genetic relationship between four wild populations (MPW, MHI, MMO and MKA) and three hatchery populations (MSH, MBH, and MTA) was due to population admixture. In Myanmar, hatchery stations frequently recruit new genetic materials from a number of fisheries and also release fingerlings into the rivers as part of the commitment to obtain a license. The releases often are made into the lower Ayeyarwaddy and its flood plains. As a result, the wild populations of Ayeyarwaddy River (MPW and MHI) are genetically similar to the MMO and MKA populations of the ThanLwin River. However, two hatchery stocks, MPY (Mandalay fisheries station) and MHL (the tributaries of the Ayeyarwaddy River) were different and grouped separately in the MDS plot. Surprisingly, all hatchery stocks displayed relatively high levels of genetic diversity comparable to wild populations. This finding suggested that these populations were useful genetic resources for establishment of breeding programs.

For selective breeding program, it is important to maintain genetic properties of the population of interest in order to minimize the effects of inbreeding and genetic drift. Therefore, the hatchery managers need to know pedigree information as well as population size of founder and current brood fish. In addition, monitoring of hatchery stocks should be regularly conducted to detect possible genetic change.

The following suggestion should be considered for successful breeding programs of mrigal. (1) Hatchery managers should conduct performance test to select the best strains that are suitable for future genetic improvement. (2)A large number of effective population sizes should be maintained by spawning at least 100 brood fish at each generation to avoid reduction of genetic variability caused by inbreeding and genetic drift. (3)Tagging system should be used even if effective population size is small to avoid closely related mating for each generation. (4)Hatchery stations can exchange founder stocks among stations to increase genetic variability and get desirable traits according to breeding goals.

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APPENDIX

Appendix Table 1 Number of sample size (n), allele number and allele frequencies at six microsatellite loci applied to eleven populations of *Cirrhinus cirrhosus* from wild and hatchery populations

Locus	Allele	Allele freq										
		MSHW	MPW	MHI	MKA	MMO	MBH	MSH	MTA	MHL	MPY	VN
Bgon22	***n***	49	49	50	50	13	42	30	44	50	50	48
	96	-	-	-	0.040	-	0.012	0.067	-	-	-	-
	99	-	0.020	-	0.020	-	-	-	-	-	-	0.031
	102	-	-	-	-	-	-	-	-	**0.010**	-	-
	105	-	-	0.020	0.010	-	0.048	-	0.011	0.080	-	0.260
	108	0.020	-	0.020	0.080	0.038	0.036	0.033	0.011	0.010	-	0.708
	111	0.010	0.153	0.130	0.130	0.192	0.179	0.150	0.205	0.230	0.190	-
	114	0.602	0.510	0.500	0.430	0.385	0.476	0.483	0.466	0.430	0.560	-
	117	0.337	0.224	0.220	0.220	0.154	0.167	0.167	0.205	0.170	0.250	-
	120	0.010	0.031	0.080	0.030	0.115	0.071	0.100	0.080	0.050	-	-
	123	0.020	0.061	0.030	0.040	0.115	0.012	-	0.023	0.020	-	-
Lr3		MSHW	MPW	MHI	MKA	MMO	MBH	MSH	MTA	MHL	MPY	VN
	n	49	49	50	50	13	42	30	44	50	50	49
	127	-	0.041	0.020	0.010	-	0.012	0.017	0.023	0.020	0.190	-
	129	-	0.010	0.010	-	-	-	-	-	-	-	-
	131	0.020	0.051	0.060	0.090	-	0.036	0.033	0.091	0.040	-	-
	133	0.837	0.551	0.550	0.540	0.692	0.667	0.467	0.682	0.600	0.750	-
	135	0.031	0.122	0.130	0.160	0.038	0.071	0.267	0.057	0.040	0.030	**1.000**
	137	0.092	0.122	0.130	0.060	0.115	0.131	0.117	0.102	0.250	0.010	-
	139	0.010	0.010	0.010	0.040	-	0.048	-	-	0.010	-	-
	141	0.010	0.031	0.060	0.100	0.154	0.036	-	-	0.020	-	-
	143	-	0.010	-	-	-	-	-	0.034	0.010	-	-
	145	-	-	-	-	-	-	-	**0.011**	-	-	-
	147	-	-	**0.010**	-	-	-	-	-	-	-	-
	151	-	**0.020**	-	-	-	-	-	-	-	-	-

Appendix Table 1 (Continued)

	153	-	-	-	-	-	-	0.067	-	-	0.010	-
	155	-	-	-	-	-	-	-	-	-	**0.010**	-
	157	-	0.020	0.010	-	-	-	0.03	-	-	-	-
	159	-	-	0.010	-	-	-	-	-	0.010	-	-
	161	-	**0.010**	-	-	-	-	-	-	-	-	-
Lr12		MSHW	MP	MHI	MKA	MMO	MBH	MSH	MTA	MHL	MPY	VN
	n	49	49	50	50	13	42	30	44	50	50	43
	151	0.020	0.031	-	0.050	0.077	0.036	-	0.045	0.010	-	-
	153	0.245	-	0.020	0.060	0.077	0.083	0.050	0.057	0.010	0.170	-
	155	0.153	0.122	0.160	0.170	0.038	0.119	0.050	0.136	0.240	0.230	-
	157	0.245	0.163	0.160	0.110	0.077	0.155	0.133	0.136	0.160	0.200	-
	159	0.071	0.122	0.120	0.070	0.154	0.083	0.083	0.102	0.070	0.110	-
	161	0.153	0.133	0.110	0.140	0.115	0.119	0.067	0.136	0.050	0.020	0.194
	163	0.010	0.031	0.040	0.030	-	0.024	0.033	0.045	0.020	-	-
	165	-	0.020	0.020	0.040	-	0.012	-	0.034	0.030	-	-
	167	-	-	0.020	0.030	0.038	0.024	-	-	-	-	0.724
	169	0.041	0.173	0.210	0.110	0.192	0.202	0.317	0.170	0.260	-	0.082
	171	0.041	0.061	0.050	0.130	0.038	0.083	0.167	0.057	0.130	0.270	-
	173	0.020	0.041	0.030	0.010	0.115	0.024	0.050	0.011	0.010	-	-
	175	-	0.010	0.010	0.010	0.077	0.024	-	0.023	-	-	-
	177	-	0.041	0.010	0.020	-	-	0.033	0.011	-	-	-
	179	-	.041	0.010	0.020	-	0.012	0.017	0.02	-	-	-
	181	-	0.010	0.020	-	-	-	-	0.011	0.010	-	-
	183	-	-	**0.010**	-	-	-	-	-	-	-	-
Lr21		MSHW	MPW	MHI	MKA	MMO	MBH	MSH	MTA	MHL	MPY	VN
	n	49	49	50	50	13	42	30	44	0	50	49
	136	-	-	0.010	0.020	-	0.024	-	-	-	-	-
	138	-	0.031	0.010	0.020	-	0.012	0.050	0.034	0.010	0.060	-
	140	-	0.194	0.140	0.180	0.192	0.095	0.233	0.193	0.060	0.300	**1.000**

Appendix Table 1 (Continued)

	142	0.959	0.561	0.690	0.530	0.615	0.583	0.500	0.648	0.660	0.600	-
	144	0.041	0.173	0.140	0.110	0.154	0.226	0.167	0.091	0.130	0.030	-
	146	-	0.010	-	0.010	-	0.024	0.033	0.023	0.130	-	-
	148	-	0.010	-	0.030	-	0.012	-	-	-	-	-
	150	-	-	0.010	-	0.038	-	0.017	0.011	0.010	-	-
	152	-	0.020	-	0.030	-	0.012	-	-	-	-	-
	154	-	-	-	-	-	0.012	-	-	-	-	-
	160	-	-	-	0.060	-	-	-	-	-	0.010	-
	164	-	-	-	**0.010**	-	-	-	-	-	-	-
MFW1		MSHW	MPW	MHI	MKA	MMO	MBH	MSH	MTA	MHL	MPY	VN
	n	49	49	50	50	13	42	30	44	50	50	45
	158	-	-	-	-	-	-	-	**0.011**	-	-	-
	160	-	-	-	-	-	-	-	**0.011**	-	-	-
	162	0.010	-	0.030	0.010	-	-	-	-	0.010	-	-
	164	0.020	0.010	0.040	0.030	-	0.012	0.033	0.011	0.010	0.010	-
	166	-	-	-	-	-	0.012	-	-	-	-	-
	168	0.020	0.041	0.010	0.030	-	0.012	0.033	0.034	0.020	0.020	0.522
	170	-	0.010	0.020	-	-	0.012	-	0.011	0.010	-	0.478
	172	0.051	0.092	0.060	0.060	0.154	0.095	0.100	0.114	0.080	-	-
	174	0.020	0.204	0.090	0.240	0.192	0.119	0.133	0.205	0.080	0.160	-
	176	0.204	0.173	0.260	0.250	0.115	0.274	0.200	0.148	0.190	0.190	-
	178	0.112	0.041	0.080	0.130	0.192	0.060	0.167	0.091	0.070	0.020	-
	180	0.133	0.163	0.140	0.080	0.154	0.143	0.050	0.080	0.260	0.190	-
	182	0.388	0.051	0.060	0.040	-	0.095	0.167	0.045	0030	0.010	-
	184	0.041	0.102	0.010	0.060	0.038	0.048	-	0.068	0.060	_	-
	186	-	0.010	0.080	0.020	0.115	0.012	-	0.045	0.010	0.060	-
	188	-	0.020	0.020	-	-	0.012	-	0.011	-	0.080	-
	190	-	0.031	0.020	0.020	-	0.036	0.067	0.023	0.030	0.210	-

Appendix Table 1 (Continued)

	192	-	0.020	0.020	-	-	-	-	0.011	0.020	0.020	-
	194	-	-	0.010	-	-	0.024	-	-	0.080	-	-
	196	-	0.010	0.040	0.010	0.038	-	-	0.023	0.02	-	-
	198	-	-	-	0.010	-	-	-	0.011	-	-	-
	200	-	-	-	-	-	0.012	0.017	0.011	-	0.020	-
	202	-	-	0.010	-	-	0.000	-	-	0.010	0010	-
	204	-	0.020	-	-	-	0.012	-	0.011	-	-	-
	208	-	-	-	-	-	-	-	**0.011**	-	-	-
	210	-	-	-	0.010	-	-	0.033	-	-	-	-
	216	-	-	-	-	-	0.012	-	-	0.010	-	-
MFW17		MSHW	MPW	MHI	MKA	MMO	MBH	MSH	MTA	MHL	MPY	VN
	n	47	49	50	47	13	40	30	44	50	50	48
	116	-	-	0.010	-	-	-	-	-	-	-	0.001
	188	-	-	-	0.043	-	-	-	-	-	-	-
	190	-	-	-	**0.011**	-	-	-	-	-	-	-
	196	-	0.010	-	0.011	-	-	-	0.011	0.010	-	-
	198	-	-	-	-	-	0.013	-	0.034	-	-	-
	200	-	0.031	0.020	-	-	0.038	-	0.023	0.030	-	-
	202	0.043	0.031	0.040	0.043	-	0.025	0.033	0.034	0.010	0.030	-
	204	-	-	-	0.021	-	0.013	-	0.023	-	-	-
	206	0.032	0061	0.040	0.032	-	0.038	0.067	0.011	0.050	0.170	-
	208	0.043	0.122	0.140	0.117	0.269	0.138	0.200	0.125	0.120	0.05	-
	210	0.245	0.245	0.240	0.234	0.115	0.150	0.117	0.193	0.170	0.300	-
	212	0.053	0.071	0.030	0.117	0.038	0.075	0.017	0.080	0.060	0.080	0.042
	214	0.223	0.082	0.100	0.064	0.115	0.038	0.150	0.080	0.020	0.010	0.344
	216	0.021	0.092	0.090	0.117	0.077	0.075	0.133	0.068	0.100	0.030	0.396
	218	-	0.020	0.060	0.043	0.115	0.075	0.017	0.068	0.030	0.040	-
	220	0.011	0.020	0.080	0.011	0.038	0.063	0.067	0.023	-	0.040	0.031
	222	0.043	0.092	0.020	0.021	-	0.125	0.067	0.068	0.210	0.250	0.188

Appendix Table 1 (Continued)

	224	-	0.010	0.010	0.021	-	0.025	-	0.011	-	-	-
	228	-	-	0.010	-	-	0.000	-	0.011	0.020	-	-
	230	0.011	0.031	-	-	-	0.013	-	0.023	-	-	-
	232	0.234	0.020	-	0.021	0.038	0.025	0.033	0.011	-	-	-
	234	0.021	-	0.010	-	-	-	-	-	-	-	-
	236	0.011	0.051	0.040	0.021	0.077	0.038	0.083	0.011	0.040	-	-
	238	-	-	-	0.021	-	0.025	-	0.045	0.010	-	-
	240	-	0.010	0.020	0.011	0.115	-	-	0.011	0.090	-	-
	242	-	-	0.020	0.011	-	0.013	-	0.011	0.020	-	-
	244	-	-	0.010	-	-	-	-	-	0.010	-	-
	246	0.11	-	0.010	0.011	-	-	0.017	-	-	-	-
	248	-	-	-	-	-	-	-	**0.01**	-	-	-

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