Spatial Genetic Structure of Thai Silver Barb *Puntius gonionotus* (Bleeker) Populations in Thailand

by

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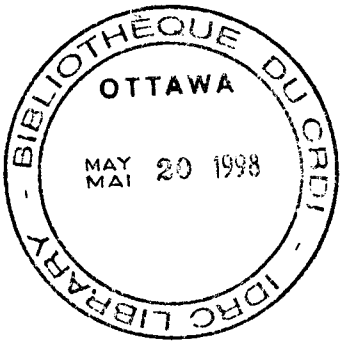

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To my parents

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ABSTRACT

Microsatellite DNA markers were developed from a Thai silver barb (*Puntius gonionotus*) genomic library and were then used to study various aspects of the genetics of *P. gonionotus* populations in Thailand. The aim is to provide means for evaluating management policies for the species in terms of conservation and genetic improvement.

Twelve natural populations from three rivers and 29 hatchery stocks from the central and north-east regions of Thailand were studied. Genetic variability was high in both groups of populations. Multidimensional scaling analysis of genetic distances revealed the discreteness apparent between watersheds among natural populations, and between geographic regions among hatchery stocks. High genetic variability within populations and significant genetic differentiation between populations both in native and hatcheries stocks indicate rich genetic resources of this species in Thailand. However, there was evidences that stock management may pose a threat of losing or altering genetic integrity of both natural and hatchery populations. Mixed stock analysis of the fish sampled from the rivers indicated 75% to 96% were from hatchery populations. This high genetic contamination of the natural populations was undoubtedly the consequence of restocking programs in which millions of *P. gonionotus* are released to rivers each year. Evidence of reduction of genetic integrity between regions was also observed in stations due to stock transfer. The results suggested an urgent need for genetically based stock management policies for both natural and hatchery populations.

The potential use of microsatellites for broodstock improvement in aquaculture was studied. Pedigrees of individuals were successfully established in a large communal rearing by using one to five microsatellites. The ability to identify individuals allowed a complicated genetic experiment and selective breeding to be conducted in places where facilities were limited. Results are considered to be more reliable because environmental variances are accounted for as fish are grown together from birth. In this study, heritability of growth traits in three stocks of *P. gonionotus* were estimated where all families were reared together. The estimates ranged from 0.193 to 0.523 suggesting that selective breeding in this species should result in good progress. However, heterozygosity in the largest individuals was greatly reduced, indicating that rapid inbreeding is very likely in simple means selection strategies.

LIST OF ABBREVIATIONS AND SYMBOLS

ANOVA	Analysis of variance
BAP	Bacterial alkaline phosphate
bp	Base pair
ddH_2O	Deionized distilled water
°C	Degree Celsius
DFREML	Derivative-free restricted maximum likelihood
DNA	Deoxyribonucleic acid
DOF	Thai Department of Fisheries
EDTA	Ethylenediaminetetraacetic acid disodium salt
h^2	Heritability
HWE	Hardy-Weinberg equilibrium
IDRC	International Development Research Centre
L	Log likelihood function
MDS	Multidimensional scaling
MGPL	Marine Gene Probe Laboratory, Dalhousie University
min	Minute
MINQUE	Minimum norm quadratic unbiased estimation
ML	Maximum likelihood
MSA	Mixed stock analysis
$N_{ec(i)}$	Inbreeding effective population size
$N_{ec(v)}$	Variance effective population size
PCR	Polymerase chain reaction
pM	pico mole ($x10^{-12}$ mole)
REML	Restricted maximum likelihood
RFLP	Restriction fragment length polymorphism
RAPD	Randomly amplified polymorphic DNA
SAS	Statistical Analysis System

SDS	Sodium dodecyl sulphate
sec	Second
SPAM	Statistics program for analyzing mixtures
SSC	Salt sodium citrate
TE	Tris EDTA
T_A	Annealing temperature
UPGMA	Unweighted pair group method with arithmetic averaging
V	Volt
VNTR	Variable number of tandem repeats
YT	Yeast extract tryptone
σ_a^2	Additive genetic variance
σ_e^2	Residual variance
σ_P^2	Total phenotypic variance

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Chapter 1

General Introduction

INTRODUCTION

Fisheries and aquaculture industries have provided inexpensive sources of animal protein to the Thai people for decades. In 1990, the total fish production (including fisheries and aquaculture) was 2,786,382 tons, amounting to a value of $2,300 million CAN (Thai Department of Fisheries, 1990). Eighty-nine percent of the total production was from capture fisheries and 11% was from aquaculture. Although fisheries has dominated the marine production (84.8% of the total production), freshwater aquaculture has become an increasingly important source of inland fish production. Freshwater aquaculture contributed 103,800 tons in 1990 which was more than 200% greater than the production in 1980. The inland fisheries production has been relatively stable during the past decade, and at about 100,000 tons a year.

The major species contributing to the freshwater production include cyprinids, catfish, gouramies and tilapias. Among these, seven species are of great importance in the Thai aquaculture industry including Nile tilapia (*Oreochromis niloticus*), walking catfish (*Clarias sp.*), silver barb (*Puntius gonionotus*), striped catfish (*Pangasius sutchi*), sepat Siam (*Trichogaster pectoralis*) and common carp (*Cyprinus carpio*). Fingerling supplies of these species come from both the private and government sectors (Thai Department of Fisheries, DOF). Fingerling production has been carried out in 56 freshwater fisheries stations around the country, and about 300 million fingerlings are produced each year. The fish produced from government hatcheries have been used for both aquaculture and restocking programs. The DOF has a target for restocking freshwater species into natural water bodies around the country of about 75 million fingerlings (in 1989) with approximately 10% increase each year (Suraswadi, 1988).

During the past several years the Thai DOF has emphasized research on improving hatchery techniques and aquaculture practices to support the rapid development of the national aquaculture industry. Little attention has been given to broodstock quality. Fish genetic research in Thailand was established in the early 1980s but not until later in the decade the research been recognized by the DOF. Genetically-based stock management has not been practiced nationwide due to a lack of basic information and practical guidelines that can be employed by fisheries stations or small farms without interference in their normal routine. Lack of genetic management will possibly lead to deleterious effects on the stocks and, when fish are produced for restocking, of the natural populations.

GENETIC DIVERSITY AND FISHERY MANAGEMENT

Genetic diversity refers to the variety of genes or hereditary units. Genetic diversity ensures the future survival of a species against the uncertain changes of environment. Sources of genetic diversity of a species are found at two fundamental levels; the differences among individuals within one population and the differences among (geographical) populations. Patterns of genetic diversity in a species are determined by both natural processes of evolution and anthropogenic processes (Bartley, 1992). Anthropogenic processes associated with development, fishing, farming and human population growth can lead to the changes of diversity pattern within a time scale of a few generations.

In the fields of fisheries and aquaculture, conservation and utilization of genetic resources are important as fish production contributes a major source of protein for world consumption. Efforts have been made to increase natural production by employing rehabilitation and restocking programs. However, the use of hatchery fish in such programs has raised concerns as it may lead to declines, in productivity and genetic diversity, in many wild stocks of fish (*e.g.*, Stickney, 1994; Waples, 1991*a*; Sheridan, 1995). Traditional hatchery practices may affect genetic resources in a number of ways including reduction in variability and vitality through founder effects or changes in the genetic structure of a stock through selection.

Founder effect and bottlenecking

Broodstock number or effective population size is an important indication of the state of genetic resources. Hatchery broodstock are often founded from a very few individuals. This small number of fish may promote both inbreeding and genetic drift that cause deleterious effects particularly on the viability and growth rate of hatchery products.

A bottleneck affects both the average heterozygosity per locus and the average number of alleles per locus. Mathematical studies by Nei *et al.* (1975) showed that the reduction in average heterozygosity per locus depends not only on the size of bottlenecks but also on the rate of population growth. When population size increases rapidly after going through a bottleneck, the reduction in average heterozygosity is rather small. The loss in average number of alleles per locus is, however, profoundly affected by bottleneck size since random drift eliminates very low frequency alleles.

Reductions of heterozygosity and number of alleles were reported in several hatchery stocks of fish and shellfish. Dillon and Manzi (1984) reported some loss of rare alleles in nursery stocks of the hard clam (*Mercenaria mercenaria*) after 4 generations of separation from the wild population. However, they could not detect any reduction in the heterozygosity of the nursery stocks. Similar results were reported on the Pacific oyster (*Crassostrea gigas*) after 3 generations of hatchery-propagation (Hedgecock & Sly, 1990).

Bottlenecking is very common in traditional hatchery practices. In Thailand, private hatcheries usually keep their broodstock to a minimum due to a limited number of ponds and to the fact that several species are produced in each farm. Government hatcheries, on the other hand, keep a fairly large number of broodstock as their aims are to produce several million fingerlings each year. Each species is produced continuously for a few months. *P. gonionotus*, for instance, is artificially bred from March to September, however, some hatcheries may collect fish only from a single batch (offspring from twenty pairs of parents or less) to replace their old broodstock every two or three years. In this case, the potential loss of genetic variability is substantial (Prakash, 1972).

Introduction of exotic stocks

Introduction means either intentional or accidental release of fish by humans into the wild (Allendorf, 1991). Introduction of exotic stocks has been practised by both aquaculturists to replace or upgrade their own stocks, and by fisheries scientists to enhance natural production. In Thailand, several million fish are released into natural

waters every year by the government's hatcheries as part of natural restoration and enhancement programs.

Introduced non-native species may cause the decline and loss of indigenous populations. Such introductions have often been accompanied by the spread of disease into new habitats, sometimes to the extreme detriment of native fish (Allendorf & Leary, 1988). Predation is another concern. The story of Lake Victoria and Nile perch is an extreme example of such problems (Tudge, 1990). Nile perch were introduced into Lake Victoria in the late 1950s with the intention of improving the lake production. The Nile perch is one of the world's supreme predators. By the mid 1980s, haplochromines, which originally accounted for 80 per cent of the fish biomass of Lake Victoria, accounted for only 1 per cent of the catch, while the perch made up 70 per cent.

Hybridisation of different gene pools by intraspecific introduction may have two important genetic consequences: loss of interpopulational genetic diversity and outbreeding depression (Waples, 1991*a*). Outbreeding depression is defined as the decline in fitness of F_1 hybrids due to genetic incompatibilities between parental stocks. Evidence has been found in plant species (Wasser & Price, 1989) but the phenomena is not yet known in fish.

Artificial selection

Artificial selection is defined as a process by which a group of individuals which are to become breeders is deliberately chosen by breeders or experimenters (Mettler & Gregg, 1969). By artificial selection, the mean of a trait under selection can be altered in whatever direction desired by the breeders. A richness of genetic variation affecting the trait in question promotes the success of artificial selection. Therefore, the mean

phenotype of a population cannot usually be changed through artificial selection in genetically uniform populations (Hartl & Clark, 1989).

The consequence of selection is the reduction of genetic variance of a trait due to alleles becoming fixed, lost or otherwise unavailable for selection. As a result, one cannot expect that the progress under artificial selection will continue forever. This limiting point is the so-called "plateau". In many experimental populations, however, the case of no response to selection is usually the result of an opposed natural selection or a lack of suitable genetic variation (Hartl & Clark, 1989).

The fear of diminishing the gene pool diversity of domestic animals by the rapid expansion of stock improvement activities and wide use of a few improved strains has been increasingly expressed (Alderson, 1989). Doyle *et al.* (1991) proposed that the genetic diversity of aquaculture stocks can be maintained while artificial selection is carried out, by the provision of strong genotype-environment interaction and locally breeding populations. As a result, genetic variation would be conserved in the among-breed components of genetic diversity. If this is the case, the fact that Thailand has more than 50 government fisheries stations countrywide will suit this strategy well.

Domestication selection

Domestication selection is defined as natural selection on traits which affect survival and reproduction in an environment that is more or less controlled by humans (Doyle, 1983). Domestication selection pressures in genetically closed aquaculture systems can be that of artificial selection and, moreover, can either support or diminish the effectiveness of artificial selection. Doyle (1983) illustrated this argument with examples from various aquaculture environments.

The consequences of domestication selection depend on how aquaculture systems are managed. In Thailand, for example, hatchery managers usually select breeders by the readiness of brooders to spawn at the time. This can result in either a positive or negative selection response depending on when in the growing cycle the brooders are picked. Illustrated by Doyle et al (1983) for giant prawn (*Macrobrachium rosenbergii*) culture that by taking brooders early in the growing cycle some considering amount of selection intensity (0.87σ) is exerted, due to a strong correlation between development rate and growth, without artificial selection. There is no doubt that it can happen in other species as well. Strong relationship between growth rate and age at maturity has been reported in several fish species, such as Atlantic salmon (Moller *et al*, 1979), common carp (Hulata *et al.*, 1974), and trout (Gall & Gross, 1978)

THESIS OBJECTIVES

This study investigated the use of genetic markers to study the structure of populations of the Thai silver barb (*Puntius gonionotus*) with the aim of providing means for developing proper management policies for the species in terms of conservation and genetic improvement.

Genetic markers offer several advantages over chemical, physical and natural markers for fish population studies. Genetic markers provide natural permanent tags that are genetically based and are heritable, thus they do not require annual re-sampling (Carvalho & Hauser, 1994). Modes of transmission of genetic markers can be clearly identified (Avise, 1994) therefore interpretation of data, which typically rely on population

genetics and evolutionary bases, can be done with relative confidence. Several genetic markers have been used in fish population studies and stock identification including allozymes, mitochondrial DNA (Lewin, 1990), restriction fragment length polymorphisms (*RFLPs*, Quinn & White, 1987), random amplified polymorphism DNA (*RAPD*, Williams *et al.*, 1990), minisatellites (Jeffreys *et al.*, 1985) and microsatellites (Tautz, 1989). Microsatellite markers were used in this study because of their several attractive properties including their ubiquitous distribution in the eukaryotic genome (Tautz & Renz, 1984) and high variability so that large number of variable loci can be developed. In addition, microsatellite analysis is done using the polymerase chain reaction (*PCR*, Saiki *et al.*, 1985) which requires only minute amounts of DNA, which can be obtained from samples such as fin-clips or scales, and allow non-invasive sampling of valuable broodstocks.

The objectives of this thesis are

(1) to develop microsatellite markers as a tool for population studies and stock identification of the Thai silver barb (*Puntius gonionotus*),

(2) to determine the existence of genetic discreteness among natural populations of *P. gonionotus* (both within- and between rivers) in Thailand,

(3) to assess genetic contamination by hatchery stocks due to the restocking of this species,

(4) to investigate genetic diversity of the hatchery stocks of *P. gonionotus* in government fisheries stations in Thailand and examine historical and cladistic relationships among stocks so that the genetic structure of the hatchery stock can be determined,

(5) to investigate the potential of parental identification in a large communal rearing using microsatellites,

(6) to assess information for genetic management of hatchery population including quantitative genetic variations, growth and survivability.

THESIS OUTLINE

Chapter 1: General introduction

Chapter 2: Development of microsatellite markers for Thai silver barb, *Puntius gonionotus* (Bleeker)

Chapter 3: Genetic structure of natural populations of *Puntius gonionotus* (Bleeker) and their management implications

Chapter 4: Spatially subdivided *Puntius gonionotus* (Bleeker) in Fisheries Stations: a potential use for genetic conservation

Chapter 5: Use of microsatellite DNA markers in genetic management of hatchery populations of *Puntius gonionotus* (Bleeker)

Chapter 6: General conclusions

Chapter 2

Development of microsatellite markers in Thai silver barb, *Puntius gonionotus (Bleeker)*

ABSTRACT

Microsatellites were isolated from a genomic library of the Thai silver barb, *Puntius gonionotus*, using a $(GT)_n$ probe. Eighty eight clones were sequenced, 39 of which contained microsatellites. They were characterized as perfect, imperfect and/or compound repeats. The common size of dinucleotide repeats was 12 copies, similar to those reported in human, canine and porcine, but smaller than those of cold-water teleost fishes. The maximum number of dinucleotide repeats was also smaller than those found in cold-water teleost fishes. PCR primers were designed for five microsatellite loci. Four loci were highly polymorphic with the average number of alleles ranging from nine to 30, and the observed heterozygosity ranging from 62.0% to 93.9%. Mendelian inheritance was verified by analysis of genotypic ratios in F_1 offspring. The results suggested that microsatellites will be valuable tools for genetic studies of population and selective breeding of Thai silver barb. In addition, four of the microsatellite primers developed from the *P. gonionotus* genomic library were successfully used to amplify homologous loci in a related taxa (*Puntius pierrei*), one of which exhibited a fixation for alternative alleles, and thus proved useful for taxonomic study of this taxa, as well as identifying their natural hybridization.

INTRODUCTION

Molecular methods have increasingly become an essential tool for genetic study of in fish populations. Molecular markers provide natural permanent tags that are genetically based and are heritable, thus they are superior to other methods; such as chemical, physical or electronic tags, meristic counts, scale patterns, parasite load (Carvalho & Hauser, 1994). Several molecular methods have been employed in fisheries, predominantly isozyme or allozyme electrophoresis (Utter, 1991). However, isozyme markers usually exhibit low variability and are not sufficient to discriminate between recently diverged populations, as is usually the case in aquaculture. The ideal markers for studies of very recent descent or kinship should possess the following: (1) many loci with high variation, (2) codominant alleles that can be unambiguously scored, and (3) ease of use (Queller *et al.*, 1993). Microsatellite markers seem to be promising to this end.

Microsatellites consist of short repeats of di, tri or tetranucleotide sequences arrayed in tandem of around 10-50 repeats (Litt & Luty, 1989; Tautz, 1989; Weber & May, 1989). Microsatellite sequences are highly abundant and dispersed randomly throughout nuclear genome of most eukaryotes (Weissenbach *et al.*, 1992; Valdes *et al.*, 1993). Their relatively high mutation rates estimated at 10^{-2} to 10^{-5} (Hearne *et al.*, 1992; Weber & Wong, 1993) cause microsatellites to exhibit extensive allelic variation and high levels of heterozygosity (Wright & Bentzen, 1994). Microsatellites provide co-dominant allelic data that can be assayed by the polymerase chain reaction (PCR), providing the flanking sequences are known (Litt & Luty, 1989; Weber & May, 1989), and size fractionated on high resolution polyacrylamide gels. Standard DNA sequencing markers,

such as a single-stranded M13 ladder (Yanisch-Perron, 1985), allow alleles to be scored consistently and unambiguously across gels. Microsatellite genotyping can also be done through automation and fluorometric detection methods (*e.g.*, Ziegle *et al.*, 1992; Kimptom *et al.*, 1993) which can improve scoring and genotyping efficiency.

To date, microsatellites have been developed in several fish species including Atlantic cod (*Gadus morhua*; Brooker *et al.*, 1994), Atlantic salmon (*Salmo salar*; Slettan *et al.*, 1993; McConnell *et al.*, 1995*a,b*), brown trout (*Salmo trutta*; Estoup *et al.*, 1993), rainbow trout (*Onchorhynchus mykiss*; Morris *et al.*, 1993, 1996), sea bass (*Dicentrarchus labrax*; García de León *et al.*, 1995), stickleback (*Gasterosteus acuelatus*; Rico *et al.*, 1993), tilapias (*Oreochromis shiranus* and *O. niloticus*; Ambali, 1996), and zebrafish (Goff *et al.*, 1992).

The objectives of the present study were to develop microsatellite markers and to determine their potential use for population studies and selective breeding of the Thai silver barb (*P. gonionotus*).

MATERIALS AND METHODS

Biological material

A blood sample from one fish was used for microsatellite development. One full-sib family of 20 offspring produced in the hatchery was analyzed for Mendelian inheritance of microsatellites. In addition, samples from four different populations including Chainat, Khonkaen, Phitsanulok and Surin (refer to Chapter 5 for details) were used to assess polymorphism and population discrimination capacities of the

microsatellites. Two types of samples were collected, i.e., blood and muscle tissue samples. About 0.5 ml of blood was drawn from individual fish using a syringe and preserved in 0.5 ml of 100% ethanol. Muscle tissue samples were collected from the anterior region below the dorsal fin of fish and preserved in 100% ethanol.

DNA extraction

For cloning purposes, nuclear DNA was extracted from preserved blood using the phenol extraction procedure (Sambrook *et al.,* 1989). Blood/ethanol suspension of 250 µl was washed twice with 1 ml high TE (100 mM Tris-Cl, 40 mM EDTA), microcentrifuged for 10 sec and the supernatant decanted. The pellet was re-suspended in 500 µl of extraction buffer (0.1 M Tris-Cl, 0.1 M EDTA, 0.25 M NaCl, 1% SDS) to which was added Proteinase K to a final concentration of 100 µg/ml. The mixture was incubated at 55 °C for 18 hours with gentle periodic agitation. Protein was removed from the cell lysate by extraction with Tris-saturated phenol (pH 8) and chloroform as follows. An equal volume of Tris-saturated phenol (500 µl) was added to the cell lysate and mixed for 10 min before subjecting to centrifugation in an Eppendorf microcentrifuge for 5 min. The aqueous phase was then transferred to a clean tube and re-extracted with 500 µl of Tris-saturated phenol. The aqueous phase was again transferred to a clean tube and extracted with an equal volume of chloroform (500 µl). The aqueous phase was transferred to a clean tube where 2 volumes of 100% cold ethanol were added and mixed to precipitated the DNA. The mixture was incubated at -20 °C for 2 hours. DNA was recovered by centrifugation in an Eppendorf microcentrifuge for 5 min at 14,000 x g and the supernatant was decanted. The DNA pellet was washed with 1 ml of cold 70% ethanol, centrifuged for 2 min and the supernatant was discarded. The pellet was dried

in a speedvac (SVC100, Savant) for 5 min. and re-suspended in 50 µl of TE (10 mM Tris-Cl, 1 mM EDTA, pH 8) overnight.

A second DNA-extraction method was performed on samples that were used for PCR amplification. A rapid extraction as described in Cook *et al.* (1996) was used. Briefly, 20 µl of blood/ethanol mixture was added to a 1.5 ml microfuge tube containing 1 ml of high TE, vortexed and centrifuged for 10 sec and the supernatant was discarded. The pellet was re-suspended in 250 µl of MGPL lysis buffer to which was added Proteinase K to a final concentration of 200 µg/ml. The mixture was incubated at 45 °C until the sample was completely digested (5-20 min with periodic agitation). Five hundred µl TE was added to the digested mixture and mixed for 10 sec. The DNA was precipitated by adding 750 µl of isopropanol, mixing well and placing at -80 °C for 20 min, DNA was recovered by centrifugation at 14,000 x g for 15 min. The pellet was washed with 1 ml 70% cold ethanol, air dried and re-suspended in 50 µl of TE at 45 °C overnight.

DNA was extracted from tissue samples by using the same procedure with the following modifications. The samples (50-100 mg) were digested for 3-5 hours (until complete dissolution of the tissue) and following dilution with TE, the solution was made up to 50 mM with sodium chloride prior to precipitation.

DNA quantity was determined by the amount of ultraviolet irradiation absorbed by the bases (Sambrook *et al.*, 1989) using a spectrophotometer (DU-64, Beckman). Samples were adjusted to a concentration of 25 ng/µl for PCR template based on uv-absorption.

Library construction

Genomic DNA from one *Puntius gonionotus* (50 μg) was digested with 100 units each of the following restriction endonucleases; *Pal*I, *Rsa*I, *Hinc*II and *Alu*I (Pharmacia) according to the manufacturer's instructions. DNA fragments were separated on 1% low melting point agarose gel for 5 hours at 40 V, with a standard molecular weight ladder (100 bp, BRL). The ladder was excised, stained with ethidium bromide and visualized under UV light. Genomic fragments of 300 to 700 bp (the lengths which are easy to sequence) were excised without staining. DNA was recovered from the gel using a phenol freeze fracture procedure (Qian & Wilkinson, 1991). In short, the segment of gel containing DNA was chopped and placed in a 15 ml corex tube and incubated at -80 °C to solidify. Three volumes of Tris-buffered saturated phenol were added and the tube was sealed with parafilm, vortexed until the chopped gel had completely dissolved. The mixture was then centrifuged in a Beckman *J2-21* centrifuge at 10,000 x g for 20 min. The upper aqueous phase was transferred to a clean 15 ml corex tube and re-extracted with Tris-saturated phenol. The aqueous phase was transferred to a clean tube and extracted with an equal volume of chloroform. The aqueous phase was placed in a clean tube containing 20 μg/ml glycogen and adjusted to 0.2 M NaCl, and added 2 volumes of 100% ethanol. The mixture was incubated at -80 °C for 30 min thereafter centrifuged in the Beckman *J2-21* centrifuge at 18,000 rpm for 30 min to pellet DNA. The supernatant was decanted and DNA was re-suspended in 0.5 ml of TE, vortexed and transferred to a 1.5 ml microfuge tube. Dissolved DNA was then re-precipitated by adding NaCl to a final concentration of 0.2 M and 2 volumes of 100% ethanol. The mixture was incubated at -80 °C for 15 min before subjecting to centrifugation in an Eppendorf microcentrifuge at 14,000 x g for 20 min. The supernatant was decanted, DNA was dried in a speedvac

and re-suspended in TE. Yield was estimated by electrophoresis of a 5 μl aliquot of the sample in a 1% agarose minigel at 90 V for 2 to 3 hours.

The size selected genomic DNA fragments (50 ng) were ligated with 40 ng of *Sma*I-digested and phosphatased pUC18 vector (Pharmacia). The ligation mix contained 1x ligation buffer (10 mM Tris-Cl pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$), 2U of T_4 DNA ligase (Pharmacia), and ddH_2O to a total volume of 25 μl. The reaction was incubated at 16 °C for 20 hours. Five μl of ligation products were transformed into 100 μl of MAX efficiency DH5α™ competent cells (BRL) according to manufacturer's recommendation. Transformed cells were plated on 2x YT medium (1.6% Bactotryptone, 1% yeast extract, 0.1 M NaCl) containing 100 μg/ml of ampicillin, and grown out at 37 °C overnight.

Library screening

Colonies were lifted onto Hybond-N nylon filters (Amersham). The filters were treated to denature and fix the DNA according to manufacturer's recommendation.

A hybridization probe was prepared by end-labeling 100 pM of a synthetic oligonucleotide [$(GT)_{15}$] with 1.5 μl of 4,500 Ci/mmol ^{32}P-γ-ATP (ICN), 5 U of T_4 polynucleotide kinase (Pharmacia), 0.5 μl of T_4 kinase buffer (Pharmacia) and 2.5 μl of sterile ddH_2O. The probe was labeled at 37 °C for 30 min., and then heated to 70 °C for 10 min. to stop enzyme activity.

The filters were incubated in pre-hybridization solution [6.25 ml 20x SSPE pH 7.6 (174 g NaCl, 27.6 g $NaH_2PO_4.H_2O$, 7.4 g EDTA and ddH_2O to a total volume of 1.0 litre), 2.5 ml 50x Denhardt's solution (5 g Ficoll-Type 400, 5 g Polyvinyl pyrrolidone, 5 g BSA (pentax fraction) and ddH_2O to a total volume of 500 ml), 0.5% SDS (w/v), 100 μl/ml

tRNA] at 65 °C for 1 hour prior to addition of heat denatured (70 °C for 5 min.) labeled probe. Hybridization was carried out at 60 °C for 12 hours in Hybaid hybridization oven.

The hybridized filters were washed three times; first with 2x SSC, 0.1% SDS for 15 min. at room temperature, second wash with 1x SSC, 0.1% SDS for 15 min. at room temperature, and final wash with 1x SSC, 0.1% SDS for at 60 °C. Filters were wrapped in Saran wrap and exposed to autoradiography film (Kodak XAR5) at -80 °C with one intensifying screen overnight.

Eighty eight individual putative positive colonies were grown up overnight in 5 ml of YT broth containing 100 μg/ml ampicillin. Plasmid DNA for sequencing were prepared using the speedprep procedure of Goode and Feinstein (1992).

DNA sequencing and primer design

Plasmid DNAs were sequenced using T7-Sequencing™ kit (Pharmacia) according to the manufacturer's instructions. Sequencing products were electrophoretically separated on an 8% denaturing polyacrylamide gel containing 7.8 M urea, and using 1x Tris-borate-EDTA buffer (90 mM Tris base, 90 mM Boric acid, 2 mM EDTA pH 8.3) at 55 mA for 2.5 hours. Sequencing gels were fixed in 10% methanol and 10% acetic acid for 1.5 hours before transferring onto Watmann 3MM paper for vacuum drying (Model 583, Bio-Rad) for 2 hours, and exposed to an autoradiography film (Kodak XAR5) overnight at room temperature.

The microsatellite-containing regions of the inserts were identified, and the unique sequences flanking the microsatellite were used to design PCR primers. The criteria used for designing primers were: (1) the primer placed as close to the repeat array as possible; (2) approximately equivalent CG content for both forward and reverse primers

and close to 50% overall; (3) containing at least one C or G at the 3' end of both primers, where possible; (4) absence of potential annealing between primers; (5) consist of 19 to 23 bp in length.

PCR amplification of microsatellites

The reverse primer of each pair of PCR primer was end-labeled with ^{32}P-γ-ATP (270 Kbq/10 pmol of primer), using T_4 polynucleotide kinase (Pharmacia). Primer labeling was performed at 37 °C for 30 min. and then at 65 °C for 15 min. to kill enzyme activity. Microsatellites were amplified from total genomic DNA (25 ng) using a PCR machine (PCT-100 Programmable Thermal Controller, MJ Research, Inc.). Reactions were carried out in microtitre plates (Costar) in 5-μl reactions, which contained 1x PCR buffer (10 mM Tris pH 8.3, 50 mM KCl, 1 mM $MgCl_2$, 0.01% gelatin), 300 μM each dNTP, 0.6 μM each of primer, 0.025 μM ^{32}P-γ-ATP labeled primer, 0.25 U Taq polymerase and 0.05% Tween20 (Kamonrat & Cook, *in prep*). Slight modifications to the reaction were made for *Pgon-17* and *Pgon-69* including: 0.05 mM ^{32}P-γ-ATP labeled primer, 0.6 mM forward primer and 0.075 μM reverse primer. Reactions were overlaid with a drop of mineral oil to prevent evaporation. Program parameters were: 6 cycles of 1 min. at 94 °C (denaturation), 30 sec at a primer specific annealing temperature (T_A) and 30 sec at 72 °C (elongation), followed by 39 cycles of 30 sec at 90 °C, 30 sec at annealing temperature and 30 sec at 72 °C.

Annealing temperature (T_A) of each pair of primers was determined by the bases content in each primer. An initial T_A was estimated for each primer as follow:

$$T_A\ (°C) = [4(\text{sum of G and C}) + 2(\text{sum of A and T})] - 6$$

T_A for each pair of primers was adjusted, after examining autoradiography film, to obtain the optimum amplification.

The amplification products were size fractionated by electrophoresis through 8% denaturing polyacrylamide gel. Five µl of stop dye (99% formamide, 0.1% bromophenol blue, 0.1% xylene cyanol green, 10 mM NaOH) was added to each of the amplification products, and denatured at 95 °C for 15 min., and 2.5 µl was loaded onto an 8% denaturing polyacrylamide gel, and immersed in 1x TBE buffer. Gels were run at 55 mA for 2 hours, fixed in 10% methanol and 10% acetic acid for 30 min, transferred onto Watmann 3MM paper for vacuum drying (Model 583, Bio-Rad) for 45 min, and exposed overnight to an autoradiography film (Kodak XAR5) at -80 °C. Microsatellite alleles were sized relative to a sequencing ladder generated from single-stranded M13 DNA (Yanisch-Perron *et al.*, 1985) loaded along side the products.

Data analysis

The informativeness of microsatellite loci were determined as follows. The number of alleles at each locus, the proportion of individuals sampled that are heterozygous (direct-count heterozygosity, h_{obs}) and the unbiased estimate of heterozygosity (h_{exp}, Nei, 1978), and were assessed for each locus using BIOSYS-1 program (Swofford & Selander, 1989). Zygotic and gametic segregation were tested against Mendelian expectation using chi-square goodness of fit test for small sample size (Sokal & Rohlf, 1981). Hardy-Weinberg expectation test for each locus in each population was a Markov chain "approximation to exact test" following Guo and Thompson (1992) and carried out using the GENEPOP program (Raymond & Rousset,

1995*a*). A sequential Bonferroni correction method was used to adjust significant levels for multiple test (Hochberg, 1988; Rice, 1989; Lessios, 1992). Fixation index (F_{is}) of each locus is the weighted averaged of F_{IS} for all alleles at a locus in each population (Wright, 1965; Nei, 1977) and was obtained from BIOSYS-1.

RESULTS and DISCUSSION

Microsatellite characteristics

A total of 88 clones were identified as putatively positive to a $(GT)_{15}$ probe and were sequenced. Thirty nine clones contained microsatellites, five of which contained two or more microsatellite regions (separated by three or more bases). Although, the library was screened for $(GT)_n$ microsatellites, which is the most abundant in vertebrates (Rafalski & Tingey, 1993), several other microsatellite motifs were also picked up by chance. Three clones contained tetranucleotide microsatellites and two clones contained trinucleotide microsatellites. Thirty six clones contained dinucleotide microsatellites, 23 of which were $(AC/GT)_n$ repeats (26.1%). Weber (1990) classified microsatellites into three categories according to the characteristic of repeat stretches including perfect, imperfect and compound repeats. Perfect repeats are uninterrupted stretches of the repeat units, while imperfect repeats contain one to three intervening bases within the stretches. Compound repeats have different motifs adjacent to each other. Most of the *P. gonionotus* microsatellites found in this study were perfect repeats (73.2%), four clones contained imperfect repeats (9.8%), and seven clones contained compound repeats (17%). The motif copy-number of microsatellites found varied from

four to 35 repeats, the most common size of dinucleotide motif was twelve, but tri- and tetranucleotide microsatellites were comprised of a smaller number of repeats. The largest motif copy-number of tri- and tetranucleotides microsatellites found were 10, and the averages were eight and seven repeats, respectively. The common size of the *P. gonionotus* microsatellites found in this study were similar to those found in higher mammals, such as humans (Weber, 1990), dog (Ostrander *et al.*, 1993) and pig (Wintero *et al.*, 1992) but was smaller than those of rainbow trout (*Onchorhynchus mykiss*; Morris *et al.*, 1996) Atlantic cod (*Gadus morhua*; Brooker *et al.*, 1994) and European flat oyster (*Ostrea edulis;* Naciri *et al.*, 1995).

Polymorphism analyses

PCR primers were designed from the unique flanking sequences of five microsatellites (Table 2.1). Four loci, *Pgon-17*, *Pgon-69*, *Pgon-75*, and *Pgon-79*, contained dinucleotide repeats and one locus, *Pgon-22*, contained trinucleotide repeats (Table 2.1). The PCR primers were tested using four unrelated populations including Chainat, Khonkaen, Phitsanulok and Surin (refer to Chapter 5 for details). The amplification products of all sets of primers produced sharp bands on the autoradiographic films that could be unambiguously scored. Four loci were highly polymorphic in the population survey. A total of 30 alleles were observed at locus *Pgon-17* with the size ranging from 107 to 181 bp, nine alleles at locus *Pgon-69* with the size ranging from 87 to 103 bp, 11 alleles at locus *Pgon-75* with the size ranging from 95 to 117 bp, and 17 alleles at locus *Pgon-79* with the size ranging from 100 to 136 bp. At the fifth locus, *Pgon-22*, only two alleles were observed in all four populations surveyed.

Distributions of allele size at four highly polymorphic loci are shown in Figures 2.1-2.4. All loci exhibited consecutive alleles that differed by multiple numbers of motifs of microsatellites except for *Pgon-69* where one allele, *allele*92*, differed by only 1 bp (2% and 6% were found in Chainat and Phitsanulok, respectively). This was unexpected from the dinucleotide-microsatellite sequence, where polymorphism is due to variation in motif copy-number. The phenomenon was also reported in the European flat oyster (Naciri *et al.*, 1995). One-base differences among microsatellite alleles may be due to deletion or insertion of a single base occurring in the unique sequences flanking the microsatellite, particularly those of which contain mononucleotide runs (D. Cook, *pers comm.*).

Although PCR amplification of microsatellites is known to generate non-amplifying alleles (null alleles) in human and other mammals we were able to detect all possible alleles (*i.e.,* 2-bp size increment) covering the size range of the four microsatellites (*Pgon-17*, *Pgon-69*, *Pgon-75* and *Pgon-79*) in our population studies (Chapter 3 and 4). Therefore it is unlikely that null alleles would be a problem in these loci.

Positive correlation between the length of microsatellite array and its variability has been reported in humans (Weber, 1990). In this study locus *Pgon-17*, which contained 34 copies of dinucleotide repeats in the original clone, exhibited the highest number of alleles (Table 2.2). The observed and expected heterozygosities were relatively high in all populations at all loci ranging from 62.0% to 93.9% (Table 2.2).

Mendelian inheritance

The Mendelian inheritance pattern of alleles of the four microsatellite loci: *Pgon-17*, *Pgon-69*, *Pgon-75* and *Pgon-79*, were studied by two methods. First was an indirect assessment by which observed variants are compared against Hardy-Weinberg expectations (HWE). Only one test, at locus *Pgon-79* of Surin, differed significantly from HWE ($P = 0.0267$) but it proved insignificance after sequential Bonferroni correction for multiple testing (Table 2.2).

The second method studied was in a full-sib family consisting of 20 progeny. For all loci, all the expected genotypes, and no unexpected ones, were observed in F_1 individuals. A significant deviation of gametic segregation from a 1:1 expectation was observed in female at *Pgon-69* (Table 2.3). The distortion was likely due to a sampling error as only 20 offspring were studied. No deviation from Mendelian expectations were observed at the genotypic frequencies.

CONCLUSIONS

The study was the first in a series of investigations on the development and use of genetic tools to study the structure of populations of the Thai silver barb (*P. gonionotus*) with the aim of providing means for assessing the proper management policies for the species in terms of conservation and genetic improvement.

Five microsatellite markers were successfully developed. The study also showed that these markers were highly polymorphic and exhibited many alleles per locus. Clearly, differences in allele frequencies between populations were observed at four loci

(*Pgon-17*, *Pgon-69*, *Pgon-75*, and *Pgon-79*) in the population survey (Fig. 2.1- 2.4). These four microsatellite loci were successfully used to identify parent-offspring relationships from a communal rearing, where several full-sib families were grown together from birth (see **chapter 5**). These results suggested that microsatellites are valuable tools for population studies and selective breeding of this species. In addition, three primers of *P. gonionotus* (*Pgon-17, Pgon-75,* and *Pgon-79*), developed in this study, amplified genomic DNA of the related taxa (*Puntius pierrei*) at which locus *Pgon-75* exhibited a "fixation of alternative alleles", where the species exhibit no common alleles at a loci (Utter & Ryman, 1993) (Fig. 2.5). *Pgon-22* was also successfully used to amplify genomic DNA of common carp (*Cyprinus carpio*; D. Morris & Y. Shi, unpublished data) and 6 species of Chinese carps including bighead carp (*Aristichthy nobilis*), silver carp (*Hypophthalmichthys molitrix*), grass carp (*Ctenopharyngodon idellus*), black carp (*Mylopharyngodon piceus*), crucian carp (*Carassius auratus*), and Wuchang fish (*Megalobrama amblycephala*) (Y. Shi & A. Ball, unpublished data) which also exhibit a fixation of alternative alleles. These findings will be useful for taxonomic studies of this taxa as well as identifying their natural hybridization.

Table 2.1 Details of the five microsatellites of *Puntius gonionotus.*

Locus	Motifs	5' - 3' Primer	Annealing temperature (°C)
Pgon-17	$(AC)_{34}$	TTACAAGGGGTTACATACTG CAGTCTCATATTTGAAAGCAG	49
Pgon-22	$(TCC)_6$	TCTTGTTGATCACACGGACG GTGACTGTACAATGAGTCTG	49
Pgon-69	$(TG)_{12}$	GCAAAGGTTCTGTCAAGG GTATCCAGAAACATGTTCAG	49
Pgon-75	$(AC)_{10}$	CTGGTAAAGACTTCAGATGC GCATGCAAAATGAGAAAGGCT	53
Pgon-79	$(CA)_{12}$	GCCAGACTGGAGCGAGG GTTCGGTGAAGCCATGAGG	53

Table 2.2 Microsatellite polymorphisms as calculated for four populations.

Population	Sample size	Number of alleles	Heterozygosity Direct count	Heterozygosity HdyWbg expected	F_{IS}
Locus: *Pgon-17*					
Chainat	50	23	0.900	0.936	+0.028
Phitsanulok	48	23	0.833	0.939	+0.096
Khonkaen	48	14	0.750	0.839	+0.103
Surin	49	20	0.775	0.826	+0.046
Locus: *Pgon-69*					
Chainat	50	7	0.640	0.691	+0.064
Phitsanulok	50	8	0.620	0.761	+0.177
Khonkaen	49	7	0.816	0.785	- 0.050
Surin	50	6	0.700	0.763	+0.074
Locus: *Pgon-75*					
Chainat	50	8	0.680	0.732	+0.062
Pitsanulok	50	8	0.660	0.765	+0.128
Khonkaen	49	9	0.877	0.783	- 0.128
Surin	50	7	0.700	0.756	+0.064
Locus: *Pgon-79*					
Chainat	50	12	0.780	0.844	+0.076
Pitsanulok	50	11	0.860	0.845	- 0.028
Khonkaen	49	9	0.673	0.649	- 0.048
Surin	50	12	0.880	0.795	- 0.118

Table 2.3 Segregation ratios of four microsatellites in 20 F_1 individuals

	Parents		Genotypes in F_1			Gametic segregation			
Locus	Female	Male	Expected	No.Observed	χ^2	Female	χ^2	Male	χ^2
Pgon-17	137/155	123/125	137/123	3					
			137/125	3					
			155/123	9					
			155/125	5	3.40^{ns}	6:14	2.45^{ns}	12:8	0.45^{ns}
Pgon-69	93/95	92/99	93/92	1					
			93/99	3					
			95/92	7					
			95/99	9	5.80^{ns}	4:16	6.05^{*}	8:12	0.45^{ns}
Pgon-75	103/103	103/103	103/103	20	--	--	--	--	--
Pgon-79	112/118	108/112	112/108	3					
			112/112	10					
			118/112	4					
			118/108	3	5.00^{ns}	14:7	1.80^{ns}	6:13	1.80^{ns}

ns = not significant at α=0.05; * $p<0.05$

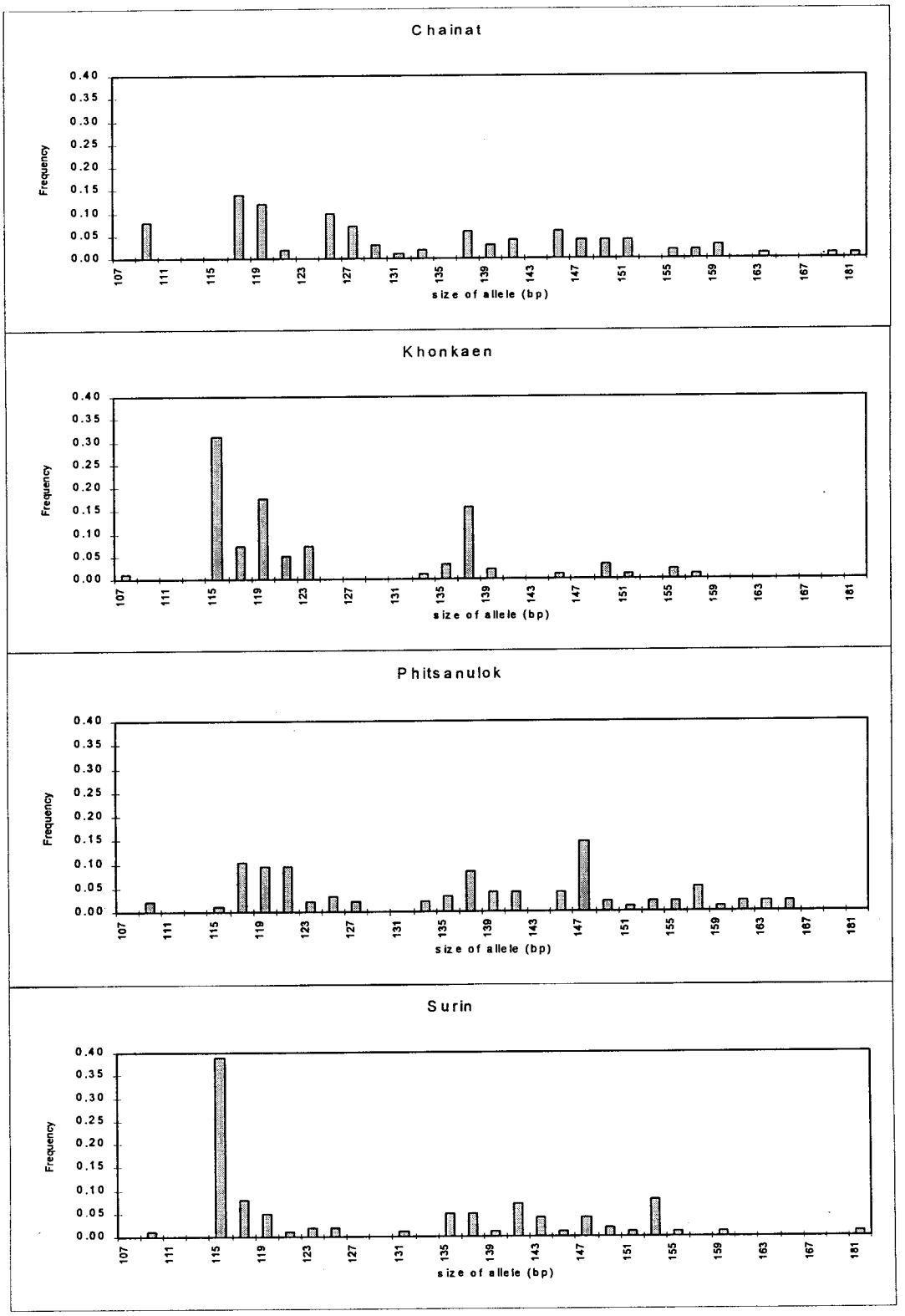


Figure 2.1 Frequency distributions of allele size at locus *Pgon-17* of *Puntius gonionotus* from four fisheries stations in Thailand

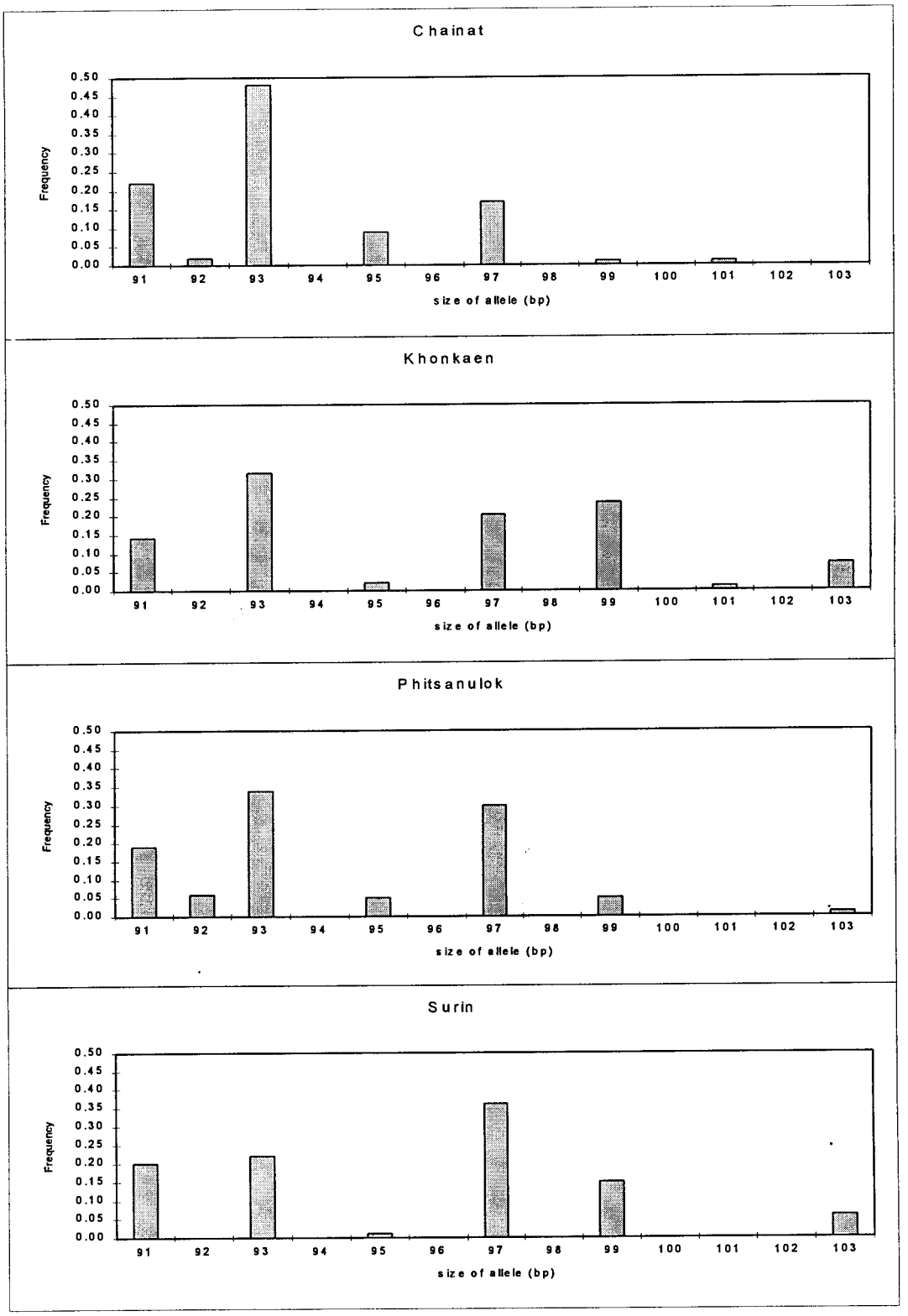


Figure 2.2 Frequency distributions of allele size at locus *Pgon-69* of *Puntius gonionotus* from four fisheries stations in Thailand.

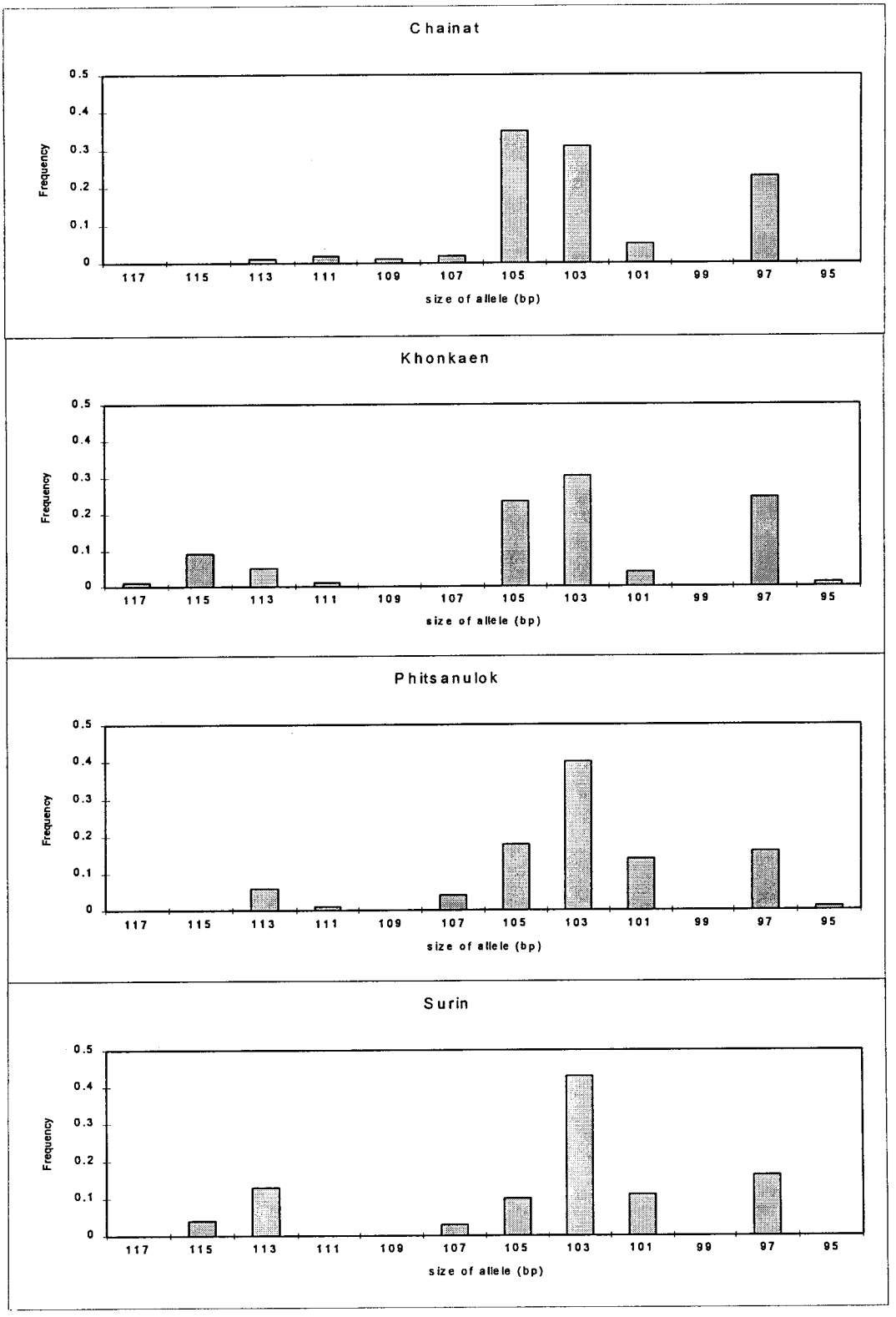


Figure 2.3 Frequency distributions of allele size at locus *Pgon-75* of *Puntius gonionotus* from four fisheries stations in Thailand.

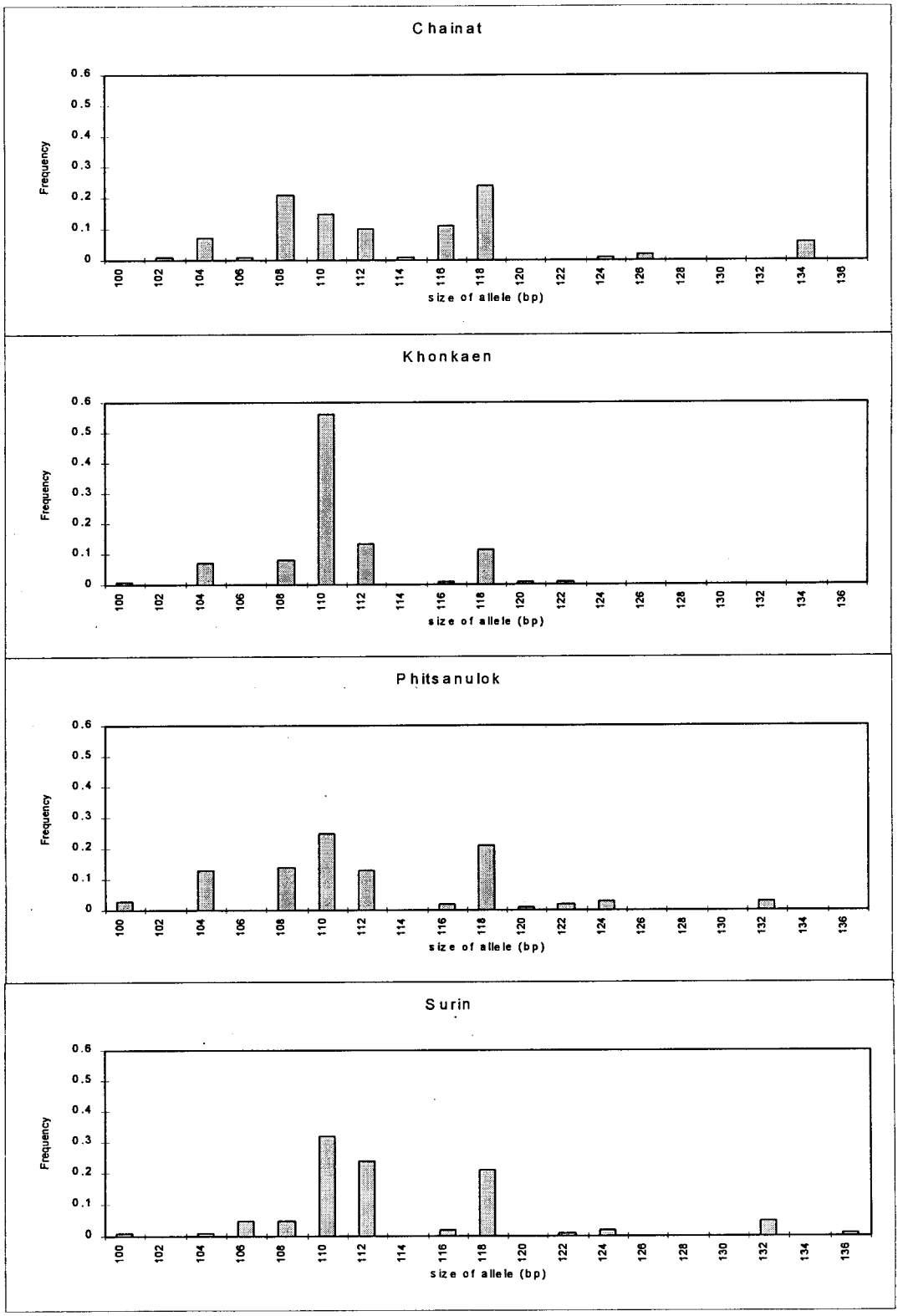


Figure 2.4 Frequency distributions of allele size at locus *Pgon-79* of *Puntius gonionotus* from four fisheries stations in Thailand.

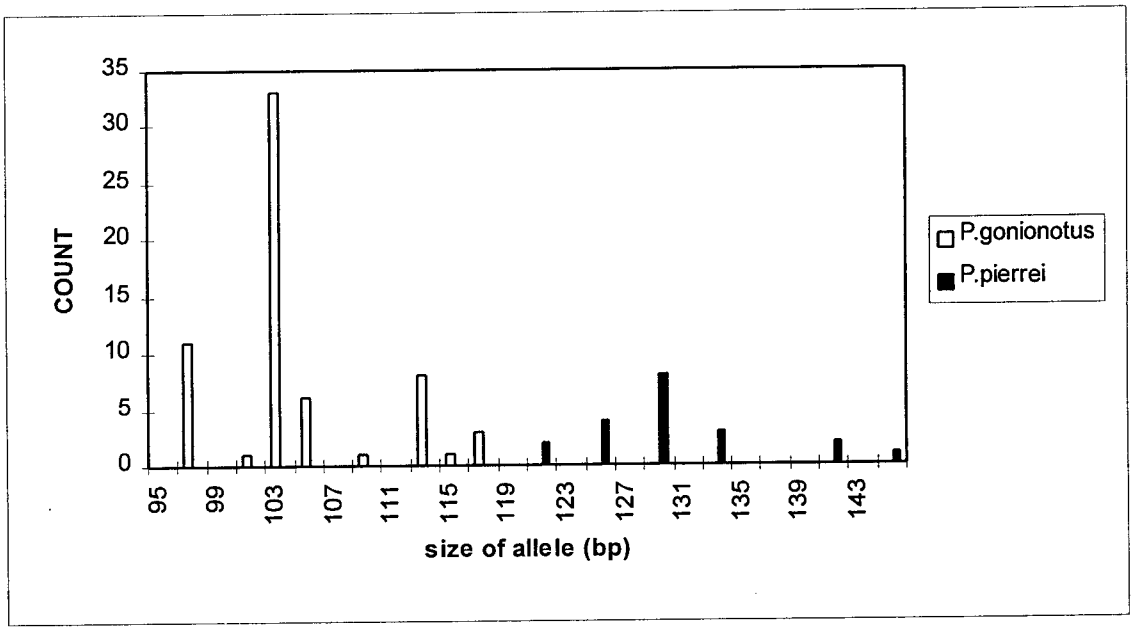


Figure 2.5 Distributions of allele sizes at locus *Pgon-75* of the two *Puntius* species (*P. gonionotus* and *P. pierrei*).

Chapter 3

Genetic structure of natural populations of *Puntius gonionotus* (Bleeker) and their management implications

ABSTRACT

Puntius gonionotus (Bleeker) is a freshwater fish inhabiting river systems in Thailand and other countries in South-east Asia. In this study, natural populations sampled from three major rivers in Thailand and nine hatchery stocks from the central region of Thailand were studied using microsatellite genetic markers. Four polymorphic microsatellite loci developed from the species were assessed and their genotypic frequencies were used to characterize population diversity and hierarchical relationships among the populations. Natural populations maintained high genetic variability with an average 9 to 12.5 alleles per locus, 3.83 to 7.03 effective number of alleles, and average heterozygosity of 0.772 to 0.844. Although the analysis suggested natural populations experienced high levels of gene flow, significant genetic differentiation was observed among populations (θ = 0.0053). A nonmetric multidimensional scaling revealed genetic discreteness between different watersheds in Thailand. Higher genetic divergence was observed between watersheds (average genetic distance of 0.265) than that of within watersheds in the central region (average genetic distance of 0.106 and 0.132 within the ChaoPhaya and MaeKlong populations, respectively). Nine hatchery populations generally possessed levels of genetic diversity similar to natural populations. However, cluster analysis and genotype frequency analysis indicated that hatchery stocks were genetically differentiated from their natural counterparts. Average genetic distances between hatchery stocks and their natural counterparts ranged from 0.117 to 0.128.

The Thai Department of Fisheries has been engaged in a restocking program to enhance natural fish production for decades. Several million fingerlings of many fish species, including *P. gonionotus*, produced by provincial fisheries stations are released into natural water bodies every year. Mixed stock analysis indicated that over 70% of the natural samples were of hatchery origins. This finding together with the evidence of genetic differentiation between hatchery fish and their natural counterparts indicated that restocking programs may pose a threat to genetic integrity of natural populations. In order to preserve the naturally existing genetic resources, it is recommended that (1) management of the natural population of this species should be devoted to different watersheds rather than individual populations, (2) specific stocks for restocking should be established from a collection of natural populations within a watershed, and (3) stock transfer must not be practiced among stations outside a watershed.

INTRODUCTION

Puntius gonionotus (Bleeker) or *P. javanicus* (Bleeker) or *Pla Tapien Khao* as it is known to the Thais, is a freshwater fish inhabiting river systems in several countries of South-east Asia, including Thailand and Indonesia. In Thailand, the species occurs throughout the country, but is most abundant in major rivers of the central region, such as ChaoPhaya, MaeKlong, NakornNayok and PaSak rivers (Smith, 1945). The fish is caught in large quantities for domestic consumption and sale. It comprised about 20 % of the total inland fishery or approximately 25,000 metric tons (DOF statistics, 1990). Although catch statistics show a relative increase of the species caught during the past decade, there is evidence of declining number of *P. gonionotus* from several locations. Many factors have contributed to its decline including overfishing, habitat loss or degradation due to industrial development, deforestation, water diversion and hydroelectric development. The Thai Department of Fisheries (DOF) has approached the problem by restocking fish into natural water bodies to enhance the natural production. The use of hatchery fish for enhancement of fisheries can, however, cause an impact on natural populations, especially when genetic integrity of the species is concerned.

Possible genetic impacts due to restocking programs depend upon how genetically different the released stocks are from the native population. Introduction of non-native populations may cause the decline or loss of the indigenous ones due to the spread of disease (Allendorf & Leary, 1988), predation (Tudge, 1990) or hybridization (Waples, 1991*a*). Released stock which originated from a native population may also cause genetic impacts to the natural population if the stock is not properly managed.

Small founder stocks, for instance, will increase inbreeding leading to the loss of genetic variation. Intentional and unintentional (e.g., domestication) selection can also cause genetic changes in response to new selective forces. Although such changes may be suitable under culture conditions, they may not be beneficial in nature. In this case, the effects may range from no detectable change due to non-breeding of the released fish, to the loss of the native population through either complete introgression or displacement (Sheridan, 1995). From a conservation point of view, differentiation among fish populations may often be adaptive and efforts should be made to identify, protect and conserve genetic discreteness among populations (FAO, 1981; Ryman, 1981; Utter, 1991; Carvalho & Hauser, 1994). A primary goal in conservation efforts is the maintenance of genetic variation within and between populations because future evolutionary adaptation depends upon the existence of genetic variation (Milligan *et al.*, 1994). Knowledge of the genetic information of fish populations is, therefore, a primary requirement for effective management of the species. Understanding of the amount and pattern of distribution of genetic variability within a species will assist in the efficient use of natural resources without any unwanted reduction of genetic variation of the species.

Although to some extent genetic variation among populations can be predicted based upon their natural habitats and life histories of the species (Wirgin & Waldman, 1994), the molecular approach provides a direct assessment of genetic variation in a population. Several molecular methods have been employed and have successfully revealed genetic differentiation among populations in several fish species. Examples include: (1) differentiation by geographical or by drainage and river systems --protein or isozyme variation, e.g., Atlantic salmon (*Salmo salar*) populations in northernmost Europe (Elo *et al.*, 1994), barramundi perch (*Lates calcarifer*) in Queensland, Australia

(Shaklee *et al.*, 1993), chinook salmon (*O. tshawytscha*) populations of California (Bartley & Gall, 1990), chum salmon (*O. keta)* in the Pacific northwest (Phelps *et al.*, 1994); --both protein and mtDNA studies, e.g., Atlantic salmon (*S. salar*) populations in Wales (O'Connell *et al.*, 1995), red drum (*Sciaenops ocellatus*), black drum (*Pogonias cromis*) and red snapper (*Lutjanus campechanus*) in the Gulf of Mexico (Gold *et al.*, 1994); and (2) temporal differentiation --protein or isozyme variation, e.g., chum salmon (*O. keta)* in the Pacific northwest (Phelps *et al.*, 1994) --mtDNA variation, e.g., females rainbow trout in a Ontario commercial farm (Danzmann *et al.*, 1994). Microsatellite DNA variation (Litt & Luty, 1989; Tautz, 1989; Weber & May, 1989) has become increasingly used in population studies of fish. Microsatellites consist of tandem arrays of short nucleotide repeats and are highly abundant in most eukaryotes genomes (Weissenbach *et al.*, 1992; Valdes *et al.*, 1993). Their high levels of polymorphism provide resolution beyond those of isozymes and prove to be informative in the study of closely related, inbred, recently separated populations or those which has experienced severe bottlenecks (Wright & Bentzen, 1994; Goldstein *et al.*, 1995).

The Thai DOF has successfully produced *P. gonionotus* fingerlings since 1960 and its culture techniques have been studied intensely. Genetic properties of the *Puntius* population have not yet, however, been studied. In the present study, *P. gonionotus* samples from several locations of the ChaoPhaya and MaeKlong rivers were examined using the microsatellite DNA markers developed from the *P. gonionotus* genomic library. The goal was to determine the existence of genetic discreteness among *P. gonionotus* populations (both within and between rivers) in Thailand. Genetic contamination of hatchery stocks due to the restocking program of the DOF was also assessed. Mixed stock analysis (Fournier *et al.*, 1984; Pella & Milner, 1987) was used to estimate the

potential contribution of hatchery populations to the natural populations in the two rivers. The results presented below suggested some levels of genetic differentiation between river systems and high proportions of hatchery populations in both rivers.

MATERIALS AND METHODS

Studied populations

The ChaoPhaya river is a major river of the central region of Thailand (Fig 3.1). The river starts from Nakornsawan after the merging of its tributaries (Ping, Wung, Yom, and Nan) from the north. From Nakornsawan, it merges with the SaKaeRung river, then the PaSak river. Then it gives a branch to the TaChean river. The ChaoPhaya river passes through many provinces including Nakornsawan, Uthaitani, Chainat, Singburi, Angthong, Ayutthaya, Pathumtani, Nontaburi, Bangkok and Samutprakarn where it drains into the Gulf of Thailand. The whole length of this river is about 380 km. The MaeKlong river is another major river of the central region located to the west (Fig. 3.1). It starts from Karnchanaburi where the KwaeYai river from Tak merges with the KwaeNoy river from Karnchanaburi. The MaeKlong River is about 130 km in length, which passes through Ratburi to the Gulf of Thailand in Samutsongkram Province.

P. gonionotus samples were obtained either directly from the rivers or from local fishermen/markets during April to August 1994 (details in Table 3.1). Seven populations were collected from the ChaoPhaya and its tributaries. Four populations were collected from the MaeKlong and its tributaries. Each population sampling site was separated by about 20 to 250 km along the rivers. Individuals collected from a common location were

treated as a random sample from that population and its name was designated by the location of sampling (Fig. 3.1). Sample sizes ranged from 43 to 60 fish. One population collected from the Mekong river was also included. However, the analyses involved mainly those populations from the ChaoPhaya and the MaeKlong rivers. A muscle tissue sample (below dorsal fin) was collected from each fish and preserved in 100% ethanol.

To determine the effects of the restocking program, samples were collected from provincial fisheries stations (seven stations from the ChaoPhaya region and two stations from the MaeKlong region) (Table 3.1). These stations are responsible for the restocking program into the rivers. Blood samples were collected from station broodstock and preserved in 100% ethanol. The sample size was 50 fish from each station.

Microsatellite analyses

Samples were DNA extracted and subjected to PCR amplification using the microsatellite primers that were developed from the *P. gonionotus* genomic library. Four polymorphic microsatelltes, namely *Pgon-17, Pgon-69, Pgon-75* and *Pgon-79*, were surveyed. DNA extraction and PCR procedures have been previously described (*see* Chapter 2). Genotypes of individuals were scored relative to a sequencing ladder generated from single-stranded M-13 DNA (Yanisch-Perron, 1985).

Statistical analyses

(a) *Nonrandom association tests:*

Two tests were carried out for testing nonrandom associations between alleles at a locus (test of Hardy-Weinberg expectations) and between alleles at different loci (test for linkage disequilibrium). Genotype frequencies were tested against Hardy-Weinberg

expectations for each locus in each population. The probability of type-I error for rejecting null hypothesis (H_0: samples are in HW equilibrium) was estimated using a Markov chain "approximation to exact test" following the algorithm of Guo and Thompson (1992). Multiple tests were combined for overall significance for each population using Fisher's combination of *P*-value method (Sokal & Rohlf, 1981).

The composite genotypic disequilibrium test (Weir, 1990*a*) was carried out for all pairs of loci in each population. The probability of type-I error for rejecting null hypothesis (H_0: population is in linkage equilibrium) was estimated using a Markov chain "approximation to exact test" followed the algorithm of Guo and Thompson (1992).

Both tests were carried out using the GENEPOP computer program (Raymond & Rousset, 1995*a*). A sequential Bonferroni correction method was used to adjust significance levels for multiple test (Hochberg, 1988; Rice, 1989; Lessios, 1992).

(b) *Within Population diversity:*

Genetic variation within each of 12 natural populations and nine fisheries stations was estimated using the following methods. Mean number of alleles per locus, the proportion of individuals sampled that are heterozygous (i.e., direct-count heterozygosity, h_{obs}) and the unbiased estimated of heterozygosity, h_{exp} (Nei, 1978) were obtained from BIOSYS-1 (Swofford & Selander, 1989). Effective number of alleles per locus was averaged over four loci, and for each locus the number of effective alleles was $1/\Sigma p_i^2$ where p_i is the *i*th allele frequency (Crow & Kimura, 1965). In addition, the fixation index of each population was calculated using Nei (1973, 1977) method as $F_{IS} = 1 - (h_{obs}/h_{exp})$.

(c) *Differentiation between populations:*

Heterogeneity of allele frequencies among populations within a river, and between station broodstock samples and natural populations were determined by the Fisher test for R x C contingency tables. The probability of type-I error (*P*-value) for rejecting null hypothesis (H_0: no differentiation among populations) was estimated using a Markov chain method (Raymond & Rousset, 1995*b*) with the GENEPOP computer program. The probabilities of all loci were combined for overall test using the Fisher's combination of *P*-value method (Sokal & Rohlf, 1981). The significant levels were adjusted for multiple tests using a sequential Bonferroni technique (Hochberg, 1988; Rice, 1989).

The amount of genetic differentiation was analyzed by using Wright's *F*-statistics approach (Wright, 1965, 1978). Values of θ (unbiased estimate of F_{ST}, Weir & Cockerham, 1984) were calculated and were tested for significance (H_0: $\theta = 0$) by the jackknifing and bootstrapping methods of Weir (1990*b*) with the computer program DIPLOIDL (listed in Weir, 1990*b*). Hierarchical *F*-statistics were also calculated for two levels (i.e., among populations within a river, and between the ChaoPhaya and MaeKlong rivers) using the method of Wright (1978) and Porter (1990). The relationship between these statistics is

$$F_{GT} = F_{ST} - \overline{F}_{SG}$$

where F_{GT} represents the component of variance attributable to differentiation among groups (rivers), $\overline{F}_{SG}$ represents the mean component of overall genetic variance attributable to populations within groups (rivers), and F_{ST} represents the component attributable to differentiation among overall populations. All *F*-statistics were obtained from BIOSYS-1 computer program (Swofford & Selander, 1989). *F*-statistics were tested

for significance (H_0: $F_{XY} = 0$) using the chi-square method of Workman and Niswander (1970): $\chi^2 = 2NF_{XY}(k-1)$ with $(k-1)(s-1)$ degree of freedom, where N is the total sample size, k is the number of alleles at the locus, and s is the number of populations.

Modified Rogers' genetic distances (Wright, 1978) were calculated between all pairs of populations and were obtained from BIOSYS-1. The pattern of genetic relationships among populations was analyzed using the multidimensional scaling (MDS) approach suggested by Lessa (1990) and carried out using SYSTAT (Wilkinson *et. al.*, 1992). The relationship between genetic and geographic distances was determined using a Mantel's test (Mantel, 1967) performed by the NTSYS-pc (Rohlf, 1993). Its significance was determined using both an approximate *t*-test and a nonparametric test with randomization of the matrices (Rohlf, 1993; Manly, 1994).

A dendogram was constructed using the unweighted pair-group method with arithmetic averages (UPGMA; Sneath & Sokal, 1973) among the populations and station stocks within a river. Modified Rogers' genetic distance was used and the dendograms were obtained from NTSYS-pc.

(d) *Estimates of stock composition:*

The estimates of stock composition were maximum likelihood estimates (MLEs) using the Statistical Program for Analyzing Mixtures (SPAM; Alaska Department of Fish and Game's genetics lab, 1995). In this procedure, two sets of data are required, one including allele frequencies of the catch mixture and a second set for the baseline stocks. Baseline stocks are the potential populations or stocks contributing to the catch mixture.

Baseline stocks and catch mixtures

In this study two groups of baseline collections were used in the stock composition estimates of samples collected from rivers in which the stations are re-stocking fish. The first group consisted of Phitsanulok (*PLSTA*), Kamphangphet (*KPSTA*), Nakornsawan (*NSSTA*), Chainat (*CNSTA*), Singburi (*SBSTA*), Angthong (*ATSTA*) and Ayutthaya (*AYSTA*) Fisheries Stations and were used for estimating the mixture in the ChaoPhaya samples. The second group consisted of Karnchanaburi (*KBSTA*) and Phetburi (*PBSTA*) Fisheries Stations and were used for estimating the mixture in the MaeKlong samples.

The ChaoPhaya samples were pooled according to their geographical proximity as follows: (1) *PLPOP-PRPOP*, (2) *KPPOP-NSPOP*, and (3) *CNPOP-SBPOP-AYPOP*. The pooling was to increase sample size, according to Wood *et al.* (1987) who suggested that a sample size of at least 40 fish per stock (*i.e.*, total of 40 x number of baseline stocks) would be required to obtain reliable MLE estimates. The MaeKlong samples consisted of *SYPOP*, *KBPOP*, *RBPOP* and *SSPOP*. These samples were not pooled as the estimation involved only 2 baseline stocks.

Optimum MLE search conditions

The performance of the MLE method depends on the number of discriminating characters used, sample size and the differences among baseline stocks. To determine the optimum conditions (*e.g.*, the number of microsatellite loci and MLE search parameters) the SPAM was first performed on a known artificial "mixture". The artificial mixture was made up as follows. The samples from each station stock (baseline stock) were divided into two groups (50% each), one of which contributed baseline data and the

other was added to the artificial "mixture". In addition, the same proportion of a distinct population (*i.e.*, *UBPOP*) was included as "unknown" stock in the artificial mixtures. This was to determine whether the MLE method would be able to detect the proportion that was not represented in the baseline collection. Adjusted baseline information (e.g., varying number of loci) and the program search parameters were made, and the conditions that yielded the nearest MLE estimates to the actual proportions in the artificial mixture were used for estimating composition of station stocks in the natural samples. The stock composition estimates are subjected to sampling errors in the genotypic composition of both the baseline stock and the mixture samples. Therefore, a bootstrap re-sampling of both the baseline and the mixture frequencies were carried out 100 times to estimate this error as recommend by Koljonen (1995).

RESULTS

Hardy-Weinberg expectations and linkage disequilibrium

Fisher's combined test for overall loci indicated no significant departure from HW expectations in the ChaoPhaya and Mekong samples. However, single-locus tests of Hardy-Weinberg expectations indicated there were occasional significant deviations from Hardy-Weinberg expectations after applying a sequential Bonferroni correction (Table 3.2). Significant deviation from HW expectations were detected in two populations (*CNPOP* and *SBPOP*) in the ChaoPhaya river and two populations (*KBPOP* and *SSPOP*) in the MaeKlong river. Four out of five significance deviations were found at

locus *Pgon-79*, and only one was found at locus *Pgon-75*. Two of MaeKlong samples (*KBPOP* and *SSPOP*) showed significant departure from HW expectation (Table 3.2).

Table 3.3 shows the results (*P*-values) of testing pairwise linkage disequilibria in each population. After applying a sequential Bonferroni correction, three populations exhibit significant nonrandom association between alleles in at least one pair of loci. The populations showing two-locus disequilibria included two (*CNPOP* and *AYPOP*) from the ChaoPhaya, and one from the MaeKlong (*KBPOP*).

Variation within natural populations

Genetic variability within population is shown in Table 3.4. All populations exhibited relatively high genetic variability and were similar both within and between the two rivers in the central region (i.e., the ChaoPhaya and the MaeKlong rivers). The MeKong population has relatively low genetic diversity. The values were likely to be slight overestimates since only highly polymorphic loci were selected during the initial development of primers. The actual mean number of alleles per locus was higher than the effective numbers since the latter takes into account the relative frequencies of alleles, to which rare alleles contribute negligibly to the estimates. The large margin of the two estimates indicate several low frequency alleles in each locus. This is probably typical for microsatellites, in which each locus displays a large number of alleles, of which many are individually rare, especially when sample sizes are small. Observed heterozygosities were lower than expected in most samples, except those from *AYPOP*, *SYPOP* and *UBPOP*. *AYPOP* exhibited the highest observed heterozygosity and differed significantly from the samples from *KPPOP* and *CNPOP* ($P > 0.05$; using an

independent *t*-test of Archie, 1985). Neither observed nor expected heterozygosities differed significantly among the MaeKlong populations.

Allele frequencies between populations within a river were very similar in both ChaoPhaya and MaeKlong rivers. There were occasional significant differentiations, most of which disappeared when a sequential Bonferroni correction was applied (Table 3.5). Among ChaoPhaya populations, significant differentiations were observed in three pairs at *Pgon-69* and one pair at *Pgon-79*, all of which involved *NSPOP*. Two significant differentiations were also observed at *Pgon-69* among MaeKlong populations, all of which involved *SYPOP*. Similar results were obtained from a Fisher's combined test over all loci (Table 3.5).

The major difference in allele frequencies between *NSPOP* and the rest of ChaoPhaya populations occurred at loci *Pgon-17*, *Pgon-69* and *Pgon-79* (Fig. 3.2): *Pgon-17*, *allele*141* was found at high frequency in *NSPOP* (0.128) but it occurred at low frequencies in others (0.023-0.068), *Pgon-69*, *allele*95* was found at high frequency in *NSPOP* (0.141) while it occurred at relatively low frequencies in others (0.011-0.051), *Pgon-79*, *allele*112* was found at a high frequency in *NSPOP* (0.265) but it occurred at relatively low frequencies in the others (0.120-0.167) while *allele*108* and *allele*110* were found at lower frequency in *NSPOP* (0.141 and 0.112, respectively) than the rest (0.156-0.300 and 0.250-0.375, respectively). Among the MaeKlong populations, the major difference in allele frequencies occurred at *Pgon-69* and *Pgon-79* (Fig. 3.2): *Pgon-69*, *allele*91* occurred at higher frequency in *SYPOP* (0.340) when compared to the rest (0.163-0.289) while *allele*97* occurred at relatively low frequency in *SYPOP* (0.110) compared to others (0.254-0.337), *Pgon-79*, *allele*118* occurred at relatively higher frequency in *SYPOP* (0.270) but lower in others (0.170-0.191).

Spatial genetic structure in natural populations

Most θ values, except those of *Pgon-17* among ChaoPhaya populations and of *Pgon-79* among MaeKlong populations, were significantly greater than zero using jackknifing methods for estimating standard error (95% CI is approximately ± 2 SE) for each locus or a bootstrap method of calculating confidence intervals over all loci (Table 3.6). All values indicate very low levels of differentiation among populations (Wright, 1978). Across all 11 populations in both rivers, the average θ was 0.0053. Although these were not significant differences between rivers, the MaeKlong populations (θ = 0.0055) tended to exhibit higher degree of differentiation than the ChaoPhaya populations (θ = 0.0035). The highest values of θ (0.0188) were detected at *Pgon-69* among four populations in the MaeKlong river. Hierarchical *F*-statistic analysis was performed to determine genetic differentiation at various levels, and the results are shown in Table 3.7. The analysis indicated that genetic differentiation between *P. gonionotus* populations in the two rivers was very low (F_{GT} = -0.001). The analysis also indicated low levels of differentiation both within rivers (F_{SG} = 0.006) and across 11 populations sampled (F_{ST} = 0.005). The highest amount of differentiation was detected at *Pgon-69* in which only 1 per cent was attributed among populations within the river (F_{SG} = 0.010). None of the *F*-statistics values were significantly different from zero, using χ^2-test (Workman & Niswander, 1970).

To determine genetic relationships among *P. gonionotus* populations, pairwise modified Rogers' genetic distances were calculated (Table 3.8A). The MeKong population (*UBPOP*) was highly divergent from the central populations. The average genetic distance between the MeKong population and those from the central populations

was 0.265. The ChaoPhaya populations were very similar while the MaeKlong populations were relatively distinct from each other. The average genetic distances were 0.106 and 0.132 in ChaoPhaya and MaeKlong populations, respectively. The average genetic distance between ChaoPhaya and MaeKlong populations was 0.116.

The global genetic relationship among the studied populations of *P. gonionotus* in the three rivers was analyzed by a nonmetric multidimensional scaling (*MDS*) of the modified Rogers' genetic distances. A four-dimensional solution was used and its corresponding stress value was 0.01478 suggesting an "excellent" fit between the configuration distances and the disparities (Rohlf, 1993). The plot of dimensions 1, 2 and 4 best revealed the genetic relationship of the populations in the rivers (Fig. 3.3). *MDS* analysis is designed to depict a complex set of relationships among samples, represented by distance or similarity matrix, in a space of a few dimensions without any significant loss of resolution (Lessa, 1990; Manly, 1994). *MDS* does not assume linearity and therefore is an appropriate analysis for most natural populations where a certain degree of gene flow occurs (Lessa, 1990).

The effect of geographical distance on genetic relationships among populations within a river was examined by plotting pairwise geographic distances against pairwise modified Rogers' genetic distances (Fig. 3.4). The points involving comparison within the ChaoPhaya river were scattered. However, among the points plotted from the MaeKlong populations there appeared to be a linear trend of gradually increasing genetic distance with increasing geographical distance. A Mantel's test of this relationship gave a high normalized Mantel's Z statistic ($r = 0.795$). The randomization test indicated a significant positive correlation between the two distance matrices, out of 24 random permutations (based on 4 populations) none were equal to or larger that the observed Z-value and the

one-tail probability of [random Z ≥ observed Z] was 0.0417 (Manley, 1994). This is consistent with the probability estimated from the approximate *t*-test(t = 1.694 and p[random Z < observed Z] = 0.955) (Rohlf, 1993).

Hatchery populations

All hatchery populations maintained relatively high genetic variability (Table 3.4). Observed heterozygosities were lower than HW expectations in most populations, except those from *KPSTA* and *KBSTA* stations. *KPSTA* samples exhibited the highest observed heterozygosity and significantly differed from the samples from *PLSTA* and *CNSTA* stations (P >0.05; using an independent *t*-test of Archie, 1985). Six stations showed low levels of heterozygote deficits (positive F_{IS}) and two stations were homozygote deficient (negative F_{IS}). The fixation index (F_{IS}) values among station broodstocks ranged from -0.0250 to 0.1016 and were relatively similar to those of natural populations.

Allele frequencies of the pooled samples from ChaoPhaya, MaeKlong, and fisheries stations are presented in Table 3.9. Generally, most alleles that occurred in the natural populations were also found in the station samples. Contingency tests of differentiation among populations indicate significant differentiation between natural populations and station stocks both within and between rivers (Table 3.10). The differences were more pronounced at 3 loci (*Pgon-17*, *Pgon-69*, and *Pgon-79*). The contingency test between station stocks and natural samples collected from the same province also indicated significant differentiation for 6 of 7 tests in the ChaoPhaya region, and 2 of 4 tests in the MaeKlong region. However, the differences were mostly attributed to *Pgon-17* and *Pgon-75* and *Pgon-79* among the ChaoPhaya populations, and to locus *Pgon-17* and *Pgon-69* among the MaeKlong populations. The average genetic distance

between the ChaoPhaya populations and its stations (0.128) is higher than that of between the MaeKlong populations and its stations (0.117).

Dendograms (*UPGMA*) constructed from the modified Rogers' genetic distance matrix indicate the differences between natural populations and station stocks (Fig. 3.5). It is worth noting that hierarchical clustering methods such as *UPGMA*, neighbor-joining, etc. does not necessarily reveal a true lineage of a species in all situations. The method relies on the assumption of hierarchical population structure. The assumption is not always appropriate for natural populations particularly where a restocking program is involved, such as the *P. gonionotus* populations in this study, or for aquaculture stocks where stocks are often established from a mixture of two or more pre-existing source populations. However, when grouping of populations is of concern, rather than their lineage, a *UPGMA* procedure may provide a good data summary as it groups populations that are most genetically similar. Its advantage is also visually appealing as a rooted tree is produced by the method. Both dendograms produced in this study confirmed well with preexisting data of stock movement among fisheries stations (a hierarchical process), and thus can be interpreted with certain degree of confidence. In the ChaoPhaya region (Fig. 3.5A), all natural populations, except *NSPOP*, were grouped together in a single major branch, which also contained *SBSTA* and *CNSTA* Fisheries stocks. All other station stocks were distributed in two branches where the stations were grouped geographically. The Nakornsawan population (*NSPOP*) was clearly separated from all samples. In the MaeKlong region, station stocks were grouped together in one major branch and the natural populations were distributed into another three major branches (Fig. 3.5B).

Stock composition estimates

Estimated proportions from artificial mixtures were close to the actual contents with relatively small differences between sample estimates (MLEs) and bootstrap estimates (Table 3.11 - 3.12). However, large differences were observed between the two estimates in the natural populations. This is probably due to the small sample size of the mixture and/or low levels of differentiation between baseline stocks. Therefore, the estimates were assessed qualitatively.

Based on the MLE search conditions that yielded the nearest estimates to the actual proportions in the artificial "mixture", the following conditions were applied for stock composition estimates of natural samples. All four loci were used in the estimation for the ChaoPhaya samples while only two loci (*Pgon-69* and *Pgon-79*) were used for the MaeKlong samples. Baseline stocks of the ChaoPhaya were pooled according to their genetic similarity. The four baseline stocks were: (1) combined *PLSTA*, *KPSTA* and *NSSTA*, (2) *CNSTA*, (3) *SBSTA*, and (4) combined *ATSTA* and *AYSTA*. Pooling baseline data will improve the accuracy of the contribution estimates when several similar (genetically) baseline stocks are used (Gall *et. al.*, 1989). No pooling was done in the MaeKlong baseline data.

The maximum likelihood estimates of proportions among the ChaoPhaya and MaeKlong samples are presented in Table 3.11 and Table 3.12, respectively. In general, station stocks accounted for a large proportion of the natural samples. Of the samples in this study, station stocks (*CPSTA*) constituted about 87% to 97 % of the natural samples from the ChaoPhaya river while the *MKSTA* constituted about 75% to 90 % of the natural samples from the MaeKlong river. The contribution of each station stock was relatively similar among all MaeKlong samples, where *KBSTA* contribution was higher (44% -

58%) than that of *PBSTA* (22% - 38%). The significant observations among the ChaoPhaya samples were as follows. The combined *PLSTA-KPSTA-NSSTA* contributed relatively large proportions (43%) in *KPPOP-NSPOP* populations and about the same proportions (25%) in *PLPOP-PRPOP* and *CNPOP-SBPOP-AYPOP* samples. *CNSTA* contributed only small proportions (1% - 7%) in the upper the ChaoPhaya river but its contribution increased in the lower region (15%). *SBSTA* contributed a very high proportion among all upper the ChaoPhaya samples (15% - 44%). The combined *ATSTA-AYSTA* contributed relatively high proportions in the lower region (39%). The unknown proportions in the MaeKlong samples (10% - 24%) were slightly higher than that of the ChaoPhaya samples (3% - 13%).

DISCUSSION

Genetic structure of Puntius gonionotus natural populations

Population genetic theory has described three models of population structure that may occur in natural populations. These are: (1) *panmixia*, where the entire population forms a single homogeneous unit due to long distance gene flow, (2) the *island model*, where gene flow occurs at an equal rate between all populations (Wright, 1978), and (3) the *isolation by distance model*, where all populations are connected by continuous migration but the rate of gene flow is greatest between neighboring populations (Wright, 1943). *P. gonionotus* inhabits a river and, thus, one would expect the most applicable model is either *panmixia* or the *isolation by distance* model depending on the geographic structure of a river. Human activities might also alter the true nature of a species by

changing geographic structure of a river, for example, by constructing dam blocking migratory pathways of fish and/or by releasing genetically different fish stocks into a river.

The *P. gonionotus* populations in Thailand are undoubtedly affected by such activities, especially by released hatchery fish. The mixed stock analysis suggests high genetic contamination by hatchery gene pools into the natural ones. This study found that there were low levels of microsatellite differentiation among *P. gonionotus* populations in both the ChaoPhaya and MaeKlong river systems. However, the statistical analyses (*F*-statistics and genetic distance) revealed evidence of geographically structured genetic variation in this species. Using an MDS analysis, genetic differentiation of the *Puntius* populations between rivers is evident (Fig. 3.3). The *UBPOP* population of the MeKong river was clearly differentiated from all populations in the Central regions. ChaoPhaya populations appeared to be very similar while MaeKlong populations were more diverse .

ChaoPhaya samples covered 470 km of the river and MaeKlong samples covered 278 km of the river. However, the MaeKlong populations exhibited stronger differentiation than those of the ChaoPhaya. A plausible explanation is the fact that along the length of 278 km of MaeKlong river, two dams have been built, Khao Laem Dam in the upper tributary (Kwae Noi river) and WaChiRaLongKorn Dam in Karnchanaburi province. These dams block the migratory pathway and thus promote differentiation between populations. Only one dam (ChaoPhaya Dam) at Sanphaya District, Chainat province is within the study range of ChaoPhaya. Large differences were observed between *NSPOP*(6) and *CNPOP*(8) of the ChaoPhaya and between *SYPOP*(15) and *KBPOP*(16) of the MaeKlong (Fig 3.3 and 3.5).

MaeKlong populations appeared to have strong geographic distance dependence. It is clear from the plot of genetic vs. geographic distance (Fig. 3.4) and Mantel's test that there was a high positive correlation between these parameters (r = 0.795). Thus, *P. gonionotus* in MaeKlong river seemed to fit well with the *isolation by distance* model. The plot of genetic vs. geographic distance does not indicate any distinct pattern among ChaoPhaya populations (Fig. 3.4). The history of these populations is known to involve founder events and mixing events (due to restocking) which are far too recent to fit the assumptions of equilibrium models such as Wright's "*isolation by distance*". Nevertheless it is interesting to study the evolution prediction of such model. Assuming the same model as the *P. gonionotus* in MaeKlong river, i.e., the *isolation by distance* model, the number of effective immigrants would be highest between adjacent populations. If this is the case, analysis using pair of adjacent populations should indicate a strong relationship between genetic and geographic distance. However, if the populations are in *panmixia*, a long-range migration would counteract such a relationship. The test was conducted based upon Slatkin's (1993) approach. The log-log plot of *Nm* (estimate from pairwise F_{ST} of Reynolds *et. al.*, 1983) and geographic distance indicated a strong linear relationship with a negative slope (Fig. 3.6) thus suggested also *isolation by distance* in ChaoPhaya populations. Pairwise F_{ST} for all natural populations are shown in Table 3.8B. Thus the model prediction (despite inappropriate assumption) gave the results consistent with the MaeKlong populations.

Evidence of admixture in natural populations

It has been recognized that most fish species are composed of discrete stocks, instead of just one single homogeneous population. Since these stocks respond to exploitation more or less independently (Carvalho & Hauser, 1994) efforts should be made to recognize discrete stocks and to identify their origins. Detecting the mixing of stocks at an intraspecific level relies on there being different frequencies of the same allele at a locus. Two tests commonly used to determine if a particular stock or catch consists of fish from more than one population are a test of Hardy-Weinberg expectations and a test for linkage disequilibrium (Utter & Ryman, 1993). Both tests rely on the population genetic principle that a mixture of gene pools will exhibit a Wahlund effect, i.e., heterozygote deficiency relative to binomial distribution. However, the HW test considers a nonrandom association between alleles at a locus, thus it is considered to be less powerful because a single-locus Wahlund effect disappears after one generation of random mating. In addition, several other factors can cause heterozygous deficiency within a single gene pool (Waples & Smouse, 1990). Linkage disequilibrium analysis, on the other hand, considers a nonrandom association between alleles at different loci. This two-locus Wahlund effect (Sinnock, 1975) decays only by the proportion of recombination between the two loci (maximum of 50% per generation for unlinked loci) (Crow, 1986). Therefore, the linkage disequilibrium may be detectable for a number of generations after the mixing event (Nei & Li, 1973).

Natural populations of *P. gonionotus* in the rivers in Thailand are subjected to both natural and artificial mixing. In the ChaoPhaya river, for example, millions of the *Puntius* fingerling from at least 7 different stations are released every year. These stations maintain their own broodstocks, some of which originated from different stocks

and already exhibit certain levels of differentiation (see Chapter 4). However, only four populations (*CNPOP*, *SBPOP*, *KBPOP* and *SSPOP*) exhibited significant deviation from HW expectations. Also, contradictory to the theoretical expectation above, only three populations (*CNPOP*, *AYPOP* and *KBPOP*) exhibited significant linkage disequilibrium.

Tests for HW expectation have long been recognized to have some unique problems. A non-randomly mating population may exhibit a genotypic frequency distribution that mimics the multinomial (Li, 1988). It is also possible for a locus under strong selection to have no apparent deviations from HW expectations (Wallace, 1958; Lewontin & Cockerham, 1959; Li, 1959; Workman, 1969). The factors that cause deviations can also pull genotype frequencies in opposite directions resulting in non-significant different between the observed and expected genotypes (Workman, 1969).

Sample size is also critical in determining the power of the test. Microsatellites generally exhibit high numbers of alleles per locus thus generating large number of possible genotypes. For example, a typical microsatellite locus having ten alleles will have 55 possible genotypes [=m(m+1)/2]. The sample size of 50, which is normally adequate for an allozyme study, is merely covering all possible genotypes generated by microsatellite loci. In this study, four out of six significant deviations from HW expectations were detected at the locus *Pgon-79* and only one was detected at *Pgon-75*. The *Pgon-79* contains 17 alleles while *Pgon-75* contains 11 alleles. Four potential significant deviations were also found at *Pgon-17*, which has 30 alleles, but none was found at *Pgon-69*, which has 9 alleles. While pooling rare alleles may be the solution, the procedure causes some loss of information of the pooled alleles.

The power of linkage disequilibrium analysis to detect mixtures of gene pools is determined by (1) the number of individuals and loci sampled, (2) the mixture

proportions, (3) genetic differences between populations, and (4) the number of generations of interbreeding since the mixing event (Waples & Smouse, 1990). Detection of linkage disequilibrium in this study was probably limited by small sample sizes and relatively great (genetic) similarity between populations within the watershed (average genetic distances were 0.106 and 0.132 among ChaoPhaya and MaeKlong populations, respectively). Waples and Smouse (1990) demonstrated that the power of detection of mixtures was 80-100% when average genetic distance is about 0.50 as compare to about 20-30% when average genetic distance was about 0.20. They also showed that unequal proportions of mixed populations can further reduce the power of detection. Many rare alleles are also found in microsatellites when the sample sizes are small. This also reduces the power of detecting significant disequilibrium (Bennett & Hsu, 1960).

Despite the evidence that admixture is undetected in several samples, the stock component analysis indicated that all populations are composed of fish from many origins. The analysis also gave clear indication of large replacement of natural populations with hatchery fish in both rivers. The high proportions of hatchery stocks (over 70%) is undoubtedly the result of the extensive restocking program of the DOF. The DOF has been practicing restocking program in all natural water bodies for decades aiming to increase natural fish production. It is roughly estimated that several million fingerlings of several species, including *P. gonionotus*, are released into both ChaoPhaya and MaeKlong rivers every year. The results also suggest that higher levels of genetic contamination occurred among populations in the ChaoPhaya river where the restocking is carried out by several fisheries stations.

Some overrepresentation was observed for the *SBSTA* stock among the upper river samples. This may be due to its genetic similarity to natural populations and, thus, it is impossible to distinguish the hatchery component from the natural one. Considering that the ChaoPhaya river is separated between upper and lower regions by the ChaoPhaya Dam, it is unlikely to find any of *SBSTA*, *ATSTA* and *AYSTA* stocks in large proportions among upper river samples. Their estimates may be accounted for as that similar component of the natural populations.

Hatchery populations vs. natural populations

All fisheries stations maintain their own broodstock but the information about their origins is scarce. It is speculated, however, that primary stocks should have been obtained from the river within the region. Most recently established stations usually obtain their stocks from the older ones. High levels of genetic diversity and low fixation index (F_{IS}) of stations samples suggested that most stations are likely to operate with relatively high effective broodstock sizes. Generally, each station keeps a broodstock size of over 300 pairs (see Chapter 4) and replacements are collected from several batches of spawning (each batch consists of 20 to 100 pairs), helping to maintain such high genetic diversity in the stocks. Although hatchery broodstocks showed levels of genetic diversity similar to those of natural populations, they were markedly divergent from each other. The difference was pronounced in different allele frequencies at various loci. There was occasionally absence of low frequency alleles from either natural or hatchery populations but this is more likely due to sampling error rather than exhibiting specific (private) alleles in the populations. Such differences may also be the effect of genetic drift when hatchery stock are established and/or replaced.

The cluster analysis clearly indicated the similarity among natural populations, except *NSPOP*, in ChaoPhaya river as oppose to the more heterogeneous group of hatchery populations (Fig. 3.5A). The result clearly indicated that station stocks on ChaoPhaya river originated from several diverse sources, some of which were highly differentiated from the natural populations and/or have undergone rapid evolution (drift) since their origin. From the ChaoPhaya dendogram (Fig. 3.5A), stations were grouped into three major branches. The northern group including *PLSTA*, *KPSTA* and *NSSTA*, was clearly distinct from natural populations. The middle group including *CNSTA* and *SBSTA*, was relatively similar to most of natural populations. The southern group, including *ATSTA* and *AYSTA*, was somewhat further distant from natural populations. The differences were likely to be related to their different broodstock origin. All stations, except *SBSTA*, have recorded that their stocks were related (by stock transfer) to each other within regional groups at some certain level (details in Chapter 4). *SBSTA* stock was unexpectedly similar to natural populations even though its broodstock consisted of a mixture of several stocks both from within the region (i.e., from *ATSTA*, whose stock is also a mixture of *AYSTA* and *SPSTA*, and private hatchery) and from the northeast (Mahasalakram Fisheries Station). Although this may be simple coincidence, it also suggested that synthetic reconstruction of a stock that is genetically similar to natural populations is possible. However, more research should be carried out to determine the stock performance in natural waterbodies.

The general goal of restocking programs is to increase natural production. Success of the program requires that hatchery fish survive after release and contributes significantly to future catches. In stocks that are genetically similar to natural populations such as *SBSTA*, a traditional mark-recapture method may be required for evaluating their performance.

In contrast to the ChaoPhaya population, the cluster analysis indicated a common origin of hatchery populations on MaeKlong river, both of which originated from its natural population (Fig 3.5B). The only record found was evidence of stock transfer from *KBSTA* to *PBSTA*, but the origin of the stock of *KBSTA* is unknown.

Management implications

The findings that (1) there are genetically distinct groups of natural *P. gonionotus* populations existing both within and between river systems in Thailand and (2) significant genetic contamination of natural catch samples by hatchery fish pose some implications for the management of this species in Thailand. The prime objectives of fisheries management are (1) to optimize the fishery production and (2) to preserve the natural existing genetic resources. To achieve these objectives one must first identify to what degree a population can be considered an evolutionary significant unit so that management can be directed to the populations most deserving of protection, particularly when resources are limited.

Dizon *et al.*(1992) suggested a phylogeographic approach in which a population is categorized as 4 categories. *Category I populations* are characterized by having large genetic differentiation and separated by strong geographic partitioning. *Category II populations* are characterized by having significant genetic diversity but with weak

geographic partitioning. *Category III populations* are the populations that have little genetic differentiation but are clearly geographically separated and likely to be reproductively isolated. *Category IV populations* are characterized by extensive gene flow and no subdivision by extrinsic barriers. They also suggested that category I populations have the highest probability of being evolutionary significant units while category IV have the lowest.

The *P. gonionotus* populations in Thailand appears to fit well with the category III populations. With the exception of the MeKong population (*UBPOP*), the differences between populations in ChaoPhaya and MaeKlong rivers were relatively small (F_{GT} = -0.001) but their differences can clearly be revealed by the *MDS* analysis. Significant genetic differentiation was also observed among populations within rivers. However, the differences are low (θ = 0.0035 and 0.0056 for ChaoPhaya and MaeKlong populations, respectively) and, thus, suggesting high levels of gene flow between populations. The stock composition analysis also confirmed the mixing of several fisheries station gene pools in all natural populations. In addition, the contingency tests of differentiation indicated most populations were similar within a river. It is, therefore, reasonable to ascribe the *P. gonionotus* populations within both the ChaoPhaya and MaeKlong rivers as category IV. This would suggest management of the natural population of this species be devoted to different watersheds rather than individual populations.

The Thai DOF has been practicing restocking for many years. Two stations, *KBSTA* and *PBSTA*, participate in restocking fish into the MaeKlong river and its tributaries while more than ten stations, seven of which were studied namely *PLSTA*, *KPSTA*, *NSSTA*, *CNSTA*, *SBSTA*, *ATSTA* and *AYSTA*, participate in restocking fish into the ChaoPhaya river and its tributaries. The fish released each year are numerous and

the mixed stock analysis indicated that hatchery fish have already contributed over 70% of the natural samples. *P. gonionotus* can easily reproduce in natural waterbodies and, thus, create high risks of genetic interaction between hatchery and natural populations. The major concerns on this issue include (Waples *et al.*, 1990) (1) the levels of genetic variability in hatchery and natural populations may differ, (2) hatchery fish may become increasingly homozygous as compared to their natural counterparts, and (3) loss of genetic integrity due to restocking genetically different stocks.

Based on the observations of this study, all hatchery populations exhibited high genetic variability that are comparable to those of natural populations. If released fish are produced from a large number of parents (e.g., 50 pairs or more) in each batch and if they are evenly released from several batches, they should pose little danger to natural populations in term of reducing their genetic variability. Managers should also realize that the number of released fish contributing to the catch is more important than the number of fish being released. Quality of released fish should be emphasized to ensure their survival in the wild. This should include the use of natural food and release of larger fish. Preserving the genetic integrity of populations is perhaps of much more important concern. As suggested above, the management of *P. gonionotus* populations in Thailand should give priority to different watersheds. The present DOF organization, in which different groups of stations are responsible to restock the different river systems, is quite effective in maintaining stock integrity at the level of the watershed. However, the genetic make-up of hatchery populations is questionable for fulfilling this role. Among all stations studied, only one station broodstock (*SBSTA*) was genetically similar to those of natural populations. Many stations also showed differentiation from their corresponding natural populations (contingency test). Replacing the existing station broodstock might

not be desirable since stations also produce fingerlings for aquaculture and their stocks might already have adapted for aquaculture conditions. It is important that fisheries stations clarify the objectives of their fingerling production, i.e., for aquaculture or for restocking programs. Establishing specific stocks for releasing purpose is recommended for stations that are responsible for restocking of the species in native watersheds. These stocks should be a collection of natural populations within a watershed. Transferring stock across watersheds, even small portions, is likely to lead to an altered genetic make up of the natives. Examples of this are of the northern group of ChaoPhaya station (*PLSTA*, *KPSTA* and *NSSTA*) part of whose stocks were either directly or indirectly transferred from the north-east region of Thailand.

Research should also be continued to identify other natural populations. Both the ChaoPhaya and MaeKlong rivers consist of numerous tributaries, the present survey has only covered the main rivers, where the restocking is intensive. Further investigation of *P. gonionotus* populations in such tributaries may lead to uncover more distinct genetic groups which are valuable resources that can be used as broodstock for both restocking and aquaculture purposes.

Table 3.1 *Puntius gonionotus* sample collections. Sample site refers to location in Fig. 3.1, *n* is sample size.

Province	Population	Abbreviation	site	*n*	Source
ChaoPhaya Region					
Phitsanulok	Phitsanulok	*PLPOP*	1	50	Nan river, Prompiram District
	Prompiram	*PRPOP*	2	50	Kawe Noi river, Prompiram District
	Hatchery	*PLSTA*	3	50	Phitsanulok Fisheries Development Center
Kamphangphet	Kamphangphet	*KPPOP*	4	43	Local market, Maung District
	Hatchery	*KPSTA*	5	50	Kamphangphet Fisheries Station
Nakornsawan	Nakornsawan	*NSPOP*	6	49	Local market, Krokphra District
	Hatchery	*NSSTA*	7	50	Nakornsawan Fisheries Development Center
Chainat	Chainat	*CNPOP*	8	50	Local fisherman, Sanphaya District
	Hatchery	*CNSTA*	9	50	Chainat Fisheries Station
Singburi	Singburi	*SBPOP*	10	50	Local fisherman, Maung District
	Hatchery	*SBSTA*	11	50	Singburi Fisheries Station
Angthong	Hatchery	*ATSTA*	12	50	Angtong Fisheries Station
Ayutthaya	Ayutthaya	*AYPOP*	13	50	ChaoPhaya river, Bangsai District
	Hatchery	*AYSTA*	14	50	Ayutthaya Fisheries Development Center
MaeKlong Region					
Karnchanaburi	Saiyok	*SYPOP*	15	50	Khaolaem Reservoir, Kwae Noi river
	Karnchanaburi	*KBPOP*	16	60	Local market, Maung District,
	Hatchery	*KBSTA*	17	50	Karnchanaburi Fisheries Development Center
Ratburi	Ratburi	*RBPOP*	18	53	Local market, Maung District
Samutsong-kram	Samutsong-kram	*SSPOP*	19	50	Local fisherman, Amphawa District
Phetburi	Hatchery	*PBSTA*	20	50	Phetburi Fisheries station
MeKong River					
Ubolratcha-thani	Ubol	*UBPOP*	21	32	Local fisherman, Khemmarat District

Table 3.2 Result of the exact test of Hardy-Weinberg proportion at the four microsatellite loci. Probabilities (*P-value*) were estimated using a Markov chain method (Guo & Thompson, 1992). The dememorization period was 1,000 steps long, number of batches was 500 and size of each batch was 1,000. Combined test was a Fisher's combination of *P*-value method (Sokal & Rohlf, 1981). Significant levels were adjusted using a sequential Bonferroni technique (Lessios, 1992).

Population	*Pgon-17*	*Pgon-69*	*Pgon-75*	*Pgon-79*	*Combined*
ChaoPhaya River					
PLPOP	0.100	0.335	0.458	0.147	0.1437
PRPOP	0.031	0.526	0.087	0.893	0.1004
KPPOP	0.042	0.732	0.212	0.700	0.2139
NSPOP	0.251	0.801	0.654	0.095	0.3629
CNPOP	0.800	0.721	0.094	0.008*	0.0482
SBPOP	0.895	0.410	0.330	0.005*	0.0639
AYPOP	0.089	0.504	0.339	0.764	0.3501
MaeKlong River					
SYPOP	0.414	0.279	0.736	0.762	0.7059
KBPOP	0.013*	0.335	0.009*	0.006*	0.0002*
RBPOP	0.725	0.885	0.138	0.014	0.0977
SSPOP	0.055	0.644	0.321	0.002*	0.0069*
MeKong River					
UBPOP	0.647	0.707	0.726	0.734	0.9456

* significant at $\alpha = 0.05$

Table 3.3 Composite genotypic linkage disequilibrium analysis for all pairs of loci in each population. The probability of type-I error (*P*-value) were estimated using a Markov chain method (Guo & Thompson, 1992). The dememorization period was 1,000 steps long, number of batches was 500 and size of each batch was 1,000. Significant levels were adjusted using a sequential Bonferroni method (Hochberg, 1988; Rice, 1989).

Population	Pgon17-69	Pgon17-75	Pgon17-79	Pgon69-75	Pgon69-79	Pgon75-79
ChaoPhaya river						
PLPOP	0.59740	0.77071	0.66535	0.61894	0.64224	0.22626
PRPOP	0.57133	0.83491	1.00000	0.96189	0.89265	0.21681
KPPOP	1.00000	1.00000	1.00000	0.47576	0.99492	0.64408
NSPOP	0.46077	1.00000	1.00000	0.81024	0.47136	0.60880
CNPOP	0.56403	1.00000	0.00273*	0.53105	0.83153	0.90589
SBPOP	0.42842	1.00000	1.00000	0.79805	0.50423	0.61233
AYPOP	1.00000	1.00000	0.35856	0.64048	0.00585*	0.97827
MaeKlong river						
SYPOP	1.00000	0.75487	1.00000	0.86758	0.36274	0.25075
KBPOP	0.21071	0.01070*	0.00464*	0.05557	0.84292	0.87188
RBPOP	1.00000	0.65228	1.00000	0.54141	0.97203	0.97180
SSPOP	1.00000	0.67368	1.00000	0.98161	0.12779	0.12105
MeKong river						
UBPOP	0.10359	0.24139	0.34700	0.95579	1.00000	0.40234

Table 3.4 Genetic variability at four loci in all populations (natural populations).

Population	Mean no. of alleles per locus	Effective number of alleles	Mean heterozygosity		F_{IS}
			Direct-count	HdyWbg expected	
ChaoPhaya River					
PLPOP	10.5±2.5	6.06	0.790±0.072	0.803±0.047	0.0162
PRPOP	9.8±2.6	5.74	0.771±0.055	0.791±0.049	0.0253
KPPOP	11.3±3.8	6.44	0.761±0.015	0.817±0.044	0.0685
NSPOP	11.0±2.9	6.71	0.775±0.028	0.820±0.043	0.0549
CNPOP	11.3±3.4	5.88	0.734±0.055	0.795±0.051	0.0767
SBPOP	11.5±4.5	6.15	0.776±0.068	0.792±0.053	0.0202
AYPOP	11.5±3.0	6.60	0.844±0.029	0.808±0.052	-0.0446
MaeKlong River					
SYPOP	11.5±4.0	6.56	0.810±0.057	0.804±0.052	-0.0075
KBPOP	12.5±3.1	6.76	0.800±0.043	0.835±0.034	0.0419
RBPOP	12.0±3.1	7.03	0.784±0.038	0.830±0.044	0.0554
SSPOP	11.8±3.1	6.71	0.722±0.077	0.805±0.054	0.1031
MeKong River					
UBPOP	9.0±1.7	3.83	0.789±0.035	0.745±0.023	-0.0591

Table 3.4 (cont) Genetic variability at four loci in all populations (fisheries stations).

Population	Mean no. of alleles per locus	Effective number of alleles	Mean heterozygosity		F_{IS}
			Direct-count	HdyWbg expected	
ChaoPhaya Region					
PLSTA	12.8±3.8	7.08	0.743±0.060	0.827±0.042	0.1016
KPSTA	12.3±2.7	6.58	0.852±0.046	0.829±0.035	-0.0277
NSSTA	10.5±2.9	5.96	0.785±0.038	0.803±0.041	0.0224
CNSTA	12.3±3.4	6.70	0.750±0.058	0.803±0.056	0.0660
SBSTA	12.0±3.5	5.65	0.800±0.078	0.800±0.047	0.0000
ATSTA	12.3±4.1	6.96	0.795±0.066	0.810±0.052	0.0185
AYSTA	11.3±3.7	6.67	0.801±0.057	0.815±0.046	0.0172
MaeKlong Region					
KBSTA	12.0±3.2	6.18	0.820±0.049	0.800±0.053	-0.0250
PBSTA	11.3±3.8	5.71	0.770±0.083	0.775±0.068	0.0065

Table 3.5 Contingency tests of differentiation among populations within a river. Probabilities of the tests were estimated using a Markov chain method (Raymon & Rousset, 1995*b*). Dememorization period was 1,000, number of batch was 100 and size of each batch was 1,000. Note that standard errors of all estimates were less than 0.01 (data not shown). Combined test (χ^2) was a Fisher's combination of *P*-value method (Sokal & Rohlf, 1981). Significant levels were adjusted using the sequential Bonferroni technique (Hochberg, 1988; Rice, 1989). Pairs populations were ordered according to their distances.

Populations	*P*-value				χ^2
	Pgon-17	*Pgon-69*	*Pgon-75*	*Pgon-79*	
ChaoPhaya River					
PLPOP - PRPOP	0.37984	0.12606	0.37227	0.15518	11.781
CNPOP - SBPOP	0.80018	0.60911	0.36043	0.06660	8.896
NSPOP - CNPOP	0.15261	0.07714	0.27798	0.00379	22.595
SBPOP - AYPOP	0.23313	0.38548	0.87463	0.11809	9.359
NSPOP - SBPOP	0.02683	0.00031*	0.09589	0*	55.714*
CNPOP - AYPOP	0.10850	0.17178	0.61429	0.14024	12.868
KPPOP - NSPOP	0.06963	0.01487	0.70820	0.27801	16.996
NSPOP - AYPOP	0.04972	0.00006*	0.32877	0.12376	31.849*
PLPOP - NSPOP	0.01288	0.00054*	0.80492	0.01343	32.807*
KPPOP - CNPOP	0.34401	0.35679	0.33878	0.84464	6.698
PRPOP - NSPOP	0.68222	0.01256	0.02408	0.01825	24.979*
KPPOP - SBPOP	0.82074	0.33688	0.13305	0.06647	12.027
PLPOP - CNPOP	0.13830	0.46048	0.49507	0.44538	8.531
PRPOP - CNPOP	0.36155	0.39315	0.03227	0.17646	14.238
PLPOP - KPPOP	0.70369	0.66936	0.99841	0.90029	1.719
PRPOP - KPPOP	0.21628	0.36719	0.24519	0.56506	9.019
PLPOP - SBPOP	0.46661	0.79573	0.20351	0.14076	9.087
PRPOP - SBPOP	0.63370	0.09491	0.03377	0.12452	16.565
KPPOP - AYPOP	0.18142	0.12346	0.29076	0.70861	10.757
PLPOP - AYPOP	0.06786	0.35291	0.54924	0.23330	11.573
PRPOP - AYPOP	0.34740	0.00596	0.15668	0.38960	17.952
MaeKlong River					
RBPOP - SSPOP	0.11670	0.29576	0.66280	0.13682	11.533
KBPOP - RBPOP	0.14238	0.14292	0.63032	0.03913	15.194
KBPOP - SSPOP	0.00696	0.05450	0.20597	0.29178	21.378*
SYPOP - KBPOP	0.01770	0.01515	0.03251	0.66824	24.106*
SYPOP - RBPOP	0.00499	0.00048*	0.20723	0.20829	32.169*
SYPOP - SSPOP	0.00992	0.00116*	0.98472	0.34132	24.926*

* significant at a = 0.05

Table 3.6 F-statistics among *Puntius gonionotus* populations in ChaoPhaya and MaeKlong rivers. Test for difference from zero using approximate 95%CI.

Locus	θ	SE	95% CI[a]
ChaoPhaya			
Pgon-17	-0.0001[NS]	0.0000	≅ -0.0001
Pgon-69	0.0041*	0.0001	(0.0039, 0.0043)
Pgon-75	0.0058*	0.0003	(0.0052, 0.0064)
Pgon-79	0.0055*	0.0002	(0.0051, 0.0059)
Overall	0.0035*	0.0015	(0.0007, 0.0056)
MaeKlong			
Pgon-17	0.0038*	0.0001	(0.0036, 0.0040)
Pgon-69	0.0188*	0.0019	(0.0150, 0.0226)
Pgon-75	0.0008*	0.0000	≅ 0.0008
Pgon-79	-0.0002[NS]	0.0000	≅ -0.0003
Overall	0.0056*	0.0042	(0.0002, 0.0151)
Both rivers			
Pgon-17	0.0016*	0.0000	≅ 0.0016
Pgon-69	0.0100*	0.0011	(0.0078, 0.0122)
Pgon-75	0.0031*	0.0001	(0.0029, 0.0033)
Pgon-79	0.0076*	0.0006	(0.0064, 0.0088)
Overall	0.0053*	0.0020	(0.0023, 0.0087)

[NS] = non-significance, * P≤ 0.05

[a] Confidence interval for each loci is θ ± 2 SE and for overall loci is derived from bootstrapped over loci, ≅ is due to SE < 0.0001

Table 3.7 Hierarchical F-statistics (Wright, 1978) among *Puntius gonionotus* populations in ChaoPhaya and MaeKlong rivers. Test for difference from zero using method of Workman and Niswander (1970).

Comparison	Locus	Variance Component	F_{XY}	χ^2
Among populations within river (SG)	Pgon-17	0.00321	0.003	87.864^{NS}
	Pgon-69	0.00734	0.010	84.800^{NS}
	Pgon-75	0.00388	0.005	54.700^{NS}
	Pgon-79	0.00352	0.004	70.912^{NS}
	Combined	**0.01794**	**0.006**	**298.276^{NS}**
Among all population (ST)	Pgon-17	0.00225	0.002	58.576^{NS}
	Pgon-69	0.00599	0.008	67.840^{NS}
	Pgon-75	0.00240	0.003	32.820^{NS}
	Pgon-79	0.00564	0.007	124.096^{NS}
	Combined	**0.01627**	**0.005**	**283.332^{NS}**
Between rivers (GT)	Pgon-17	-0.00096	-0.001	--
	Pgon-69	-0.00135	-0.002	--
	Pgon-75	-0.00148	-0.002	--
	Pgon-79	0.00211	0.003	53.184^{NS}
	Combined	**-0.00167**	**-0.001**	**--**

^{NS} not significant different

Table 3.8 Geographic distances in km. (above diagonal) and modified Rogers's genetic distances (below diagonal) among *Puntius gonionotus* populations in ChaoPhaya and MaeKlong rivers (3.8A) and pairwise F_{ST} (3.8B).

	PL	PR	KP	NS	CN	SB	AY	SY	KB	RB	SS
PL	--	33	340	239	324	362	455	297	339	385	392
PR	0.099	--	355	254	339	377	470	294	330	377	385
KP	0.074	0.094	--	165	250	288	381	210	270	324	338
NS	0.134	0.144	0.113	--	85	123	216	180	180	223	234
CN	0.071	0.109	0.080	0.117	--	38	131	175	146	184	191
SB	0.088	0.118	0.105	0.152	0.092	--	93	196	130	158	164
AY	0.099	0.118	0.105	0.124	0.094	0.097	--	223	105	104	102
SY	0.138	0.144	0.139	0.137	0.136	0.130	0.092	--	170	257	278
KB	0.123	0.125	0.097	0.104	0.115	0.132	0.112	0.113	--	87	108
RB	0.120	0.129	0.096	0.120	0.106	0.121	0.113	0.142	0.088	--	21
SS	0.095	0.105	0.100	0.131	0.096	0.089	0.112	0.123	0.109	0.105	--

(Table 3.8A)

	PL	PR	KP	NS	CN	SB	AY	SY	KB	RB	SS
PL	--										
PR	0.002	--									
KP	-0.004	0	--								
NS	0.012	0.015	0.005	--							
CN	-0.004	0.005	-0.003	0.007	--						
SB	0	0.007	0.003	0.018	0.001	--					
AY	0.002	0.007	0.003	0.009	0.001	0.002	--				
SY	0.013	0.015	0.013	0.013	0.013	0.011	0.001	--			
KB	0.009	0.010	0.002	0.004	0.007	0.012	0.006	0.006	--		
RB	0.008	0.011	0.001	0.008	0.004	0.008	0.006	0.015	0	--	
SS	0.001	0.003	0.002	0.011	0.002	0	0.006	0.009	0.005	0.004	--

(Table 3.8B)

Table 3.9 Allele frequencies at each locus in natural and station broodstock samples. *CPPOP* = ChaoPhaya populations, *CPSTA* = Fisheries Stations in ChaoPhaya region, *MKPOP* = MaeKlong population, *MKSTA* = Fisheries Stations in MaeKlong region.

Locus	Allele	*CPPOP*	*CPSTA*	*MKPOP*	*MKSTA*
Pgon-17	**109*	0	0.0412	0.0165	0.0750
	**115*	0.0081	0.0074	0.0189	0.0050
	**117*	0.0855	0.1059	0.0849	0.0600
	**119*	0.1516	0.1206	0.1722	0.1800
	**121*	0.0871	0.0485	0.0991	0.1450
	**123*	0.0355	0.0132	0.0283	0
	**125*	0.0516	0.0500	0.0283	0.0350
	**127*	0.0242	0.0368	0.0283	0.0300
	**129*	0.0016	0.0088	0.0024	0
	**131*	0.0032	0.0103	0.0047	0.0150
	**133*	0.0081	0.0059	0.0142	0.0050
	**135*	0.0081	0.0191	0.0142	0.0200
	**137*	0.0629	0.0735	0.0519	0.0450
	**139*	0.0355	0.0412	0.0354	0.0400
	**141*	0.0613	0.0353	0.0354	0.0150
	**143*	0.0097	0.0059	0.0236	0
	**145*	0.0435	0.0574	0.0708	0.0700
	**147*	0.0758	0.0912	0.0613	0.0600
	**149*	0.0565	0.0588	0.0566	0.0750
	**151*	0.0613	0.0338	0.0377	0.0400
	**153*	0.0242	0.0265	0.0330	0.0050
	**155*	0.0371	0.0221	0.0283	0.0200
	**157*	0.0242	0.0265	0.0118	0.0050
	**159*	0.0113	0.0132	0.0212	0.0200
	**161*	0.0161	0.0132	0.0047	0.0200
	**163*	0.0081	0.0147	0.0024	0.0100
	**165*	0.0016	0.0147	0.0047	0.0050
	**167*	0	0	0	0
	**171*	0	0.0015	0.0071	0
	**181*	0.0065	0.0029	0.0024	0

Table 3.9 (cont.)

Pgon-69	**85*	0.0085	0.0043	0.0075	0
	**91*	0.2288	0.2197	0.2613	0.2653
	**92*	0.0413	0.0592	0.0704	0.0561
	**93*	0.4184	0.4118	0.3492	0.5102
	**95*	0.0392	0.0477	0.0377	0.0051
	**97*	0.2426	0.2124	0.2487	0.1582
	**99*	0.0159	0.0275	0.0050	0
	**101*	0.0021	0.0014	0.0151	0.0051
	**103*	0.0032	0.0145	0.0050	0
	**105*	0	0.0014	0	0
Pgon-75	**95*	0.0015	0.0029	0.0024	0.0051
	**97*	0.1794	0.2040	0.1860	0.1869
	**101*	0.0809	0.0963	0.0773	0.0505
	**103*	0.3500	0.3175	0.3237	0.4040
	**105*	0.2706	0.2687	0.2995	0.2475
	**107*	0.0265	0.0129	0.0290	0.0051
	**109*	0.0059	0.0014	0	0.0101
	**111*	0.0103	0.0187	0.0169	0.0202
	**113*	0.0750	0.0661	0.0652	0.0657
	**115*	0	0.0057	0	0.0051
	**117*	0	0.0057	0	0
Pgon-79	**100*	0.0117	0.0330	0.0399	0.0051
	**102*	0	0.0057	0.0023	0
	**104*	0.0572	0.0616	0.0423	0.0455
	**106*	0	0.0014	0.0188	0.0101
	**108*	0.2859	0.2049	0.1808	0.2020
	**110*	0.1994	0.2163	0.1596	0.1970
	**112*	0.1569	0.1490	0.1901	0.1768
	**114*	0.0015	0.0115	0.0094	0.0051
	**116*	0.0367	0.0387	0.0446	0.0556
	**118*	0.1716	0.1705	0.1995	0.1919
	**120*	0.0147	0.0158	0.0070	0
	**122*	0.0249	0.0086	0.0258	0.0303
	**124*	0.0132	0.0330	0.0563	0.0455
	**126*	0	0.0086	0.0047	0
	**128*	0.0029	0	0	0.0051
	**130*	0	0	0	0.0101
	**132*	0.0205	0.0201	0.0117	0.0051
	**134*	0.0029	0.0186	0.0047	0.0051
	**136*	0	0.0014	0.0023	0.0101
	**138*	0	0.0014	0	0

Table 3.10 Contingency tests of differentiation among populations between station stocks and their natural populations. Probabilities of the tests were estimated using a Markov chain method (Raymond & Rousset, 1995*b*). Dememorization period was 1,000, number of batch was 100 and size of each batch was 1,000. Note that standard errors of all estimates were less than 0.01 (data not shown). Significant levels were adjusted using a sequential Bonferroni (Hochberg, 1988; Rice, 1989).

Populations	*Pgon-17*	*Pgon-69*	*Pgon-75*	*Pgon-79*	χ^2
Combined [a]					
CPPOP-MKPOP	0.00288*	0.03744	0.77323	0*	46.415*
CPSTA-CPPOP	0*	0.15571	0.06164	0*	64.554*
MKSTA-MKPOP	0.00624	0.00119*	0.06664	0.27372	31.629*
CPSTA-MKSTA	0.00254*	0.00092*	0.16765	0.00220*	41.744*
CPSTA-MKPOP	0.00007*	0.00269*	0.33734	0.00171*	45.886*
MKSTA-CPPOP	0*	0.00360*	0.13058	0.00177*	55.630*
ChaoPhaya river					
PLSTA - PLPOP	0.14554	0.52303	0.72040	0.07546	10.975
PLSTA - PRPOP	0.17410	0.12620	0.03029	0.00357*	25.900*
KPSTA - KPPOP	0.07566	0.99171	0.08123	0.00010*	28.621*
NSSTA - NSPOP	0.00462*	0.04855	0.00414*	0*	55.410*
CNSTA - CNPOP	0.02240	0.79124	0.13398	0.00507*	22.655*
SBSTA - SBPOP	0.24683	0.32132	0.02131	0.00141*	25.894*
AYSTA - AYPOP	0.00102*	0.93876	0.03374	0.12296	24.872*
MaeKlong river					
KBSTA - SYPOP	0.00289*	0.12057	0.21774	0.97235	19.029*
KBSTA - KBPOP	0.11490	0.03113	0.25389	0.58360	15.085
PBSTA - RBPOP	0.05222	0*	0.64269	0.12522	38.575*
PBSTA - SSPOP	0.01890	0.04200	0.68663	0.63690	15.932

[a] combined all populations or stations of ChaoPhaya (CP) and MaeKlong (MK)
* significant at $\alpha = 0.05$

Table 3.11 Maximum likelihood estimates (MLE) of proportions of different stocks, bootstrapped estimations and standard deviations resulting from resampling (100 times) of mixture and baseline data for ChaoPhaya samples. Actual proportions in the artificial mixture are shown in parentheses.

Sample population	n	Baseline stock	MLE	Bootstrap estimate
Artificial mixture	160	*PLSTATION*[a]	0.3624 (0.375)	0.3516±0.0764
		CNSTATION	0.0893 (0.125)	0.0974±0.0646
		SBSTATION	0.1643 (0.125)	0.1621±0.0704
		AYSTATION[b]	0.2582 (0.250)	0.2374±0.0000
		Unknown	0.1258 (0.125)	0.1516
Upper region (*PLPOP-PRPOP*)	98	*PLSTATION*[a]	0.2540	0.2881±0.1105
		CNSTATION	0.0786	0.0703±0.0560
		SBSTATION	0.4462	0.2922±0.1072
		AYSTATION[b]	0.1906	0.2569±0.0000
		Unknown	0.0306	0.0926
Upper region (*KPPOP-NSPOP*)	93	*PLSTATION*[a]	0.4342	0.4061±0.1007
		CNSTATION	0.0035	0.0448±0.0499
		SBSTATION	0.2997	0.1996±0.0784
		AYSTATION[b]	0.1336	0.1321±0.0000
		Unknown	0.1290	0.2175
Lower region (*CNPOP-SBPOP-AYPOP*)	156	*PLSTATION*[a]	0.2639	0.2240±0.0911
		CNSTATION	0.1492	0.1801±0.1383
		SBSTATION	0.1478	0.2273±0.1298
		AYSTATION[b]	0.3945	0.2982±0.0000
		Unknown	0.0446	0.0704

[a] *PLSTATION* = combined *PLSTA*, *KPSTA* and *NSSTA*
[b] *AYSTATION* = combined *ATSTA* and *AYSTA*

Table 3.12 Maximum likelihood estimates (MLE) of proportions of different stocks, bootstrapped estimations and standard deviations resulting from resampling (100 times) of mixture and baseline data for MaeKlong samples. Actual proportions in the artificial mixture are shown in parentheses.

Sample population	n	Baseline stock	MLE	Bootstrap estimate
Artificial mixture	75	*KBSTATION*	0.4008 (0.333)	0.3997±0.1164
		PBSTATION	0.2926 (0.333)	0.2835±0.0000
		Unknown	0.3067 (0.333)	0.3168
SYPOP	50	*KBSTATION*	0.4490	0.4536±0.1369
		PBSTATION	0.3310	0.2920±0.0000
		Unknown	0.2200	0.2544
KBPOP	53	*KBSTATION*	0.5296	0.4627±0.1415
		PBSTATION	0.2251	0.2409±0.0000
		Unknown	0.2453	0.2964
RBPOP	53	*KBSTATION*	0.4969	0.4156±0.1423
		PBSTATION	0.3899	0.4277±0.0000
		Unknown	0.1132	0.1567
SSPOP	50	*KBSTATION*	0.5811	0.5783±0.1571
		PBSTATION	0.3189	0.3189±0.0000
		Unknown	0.1000	0.1028

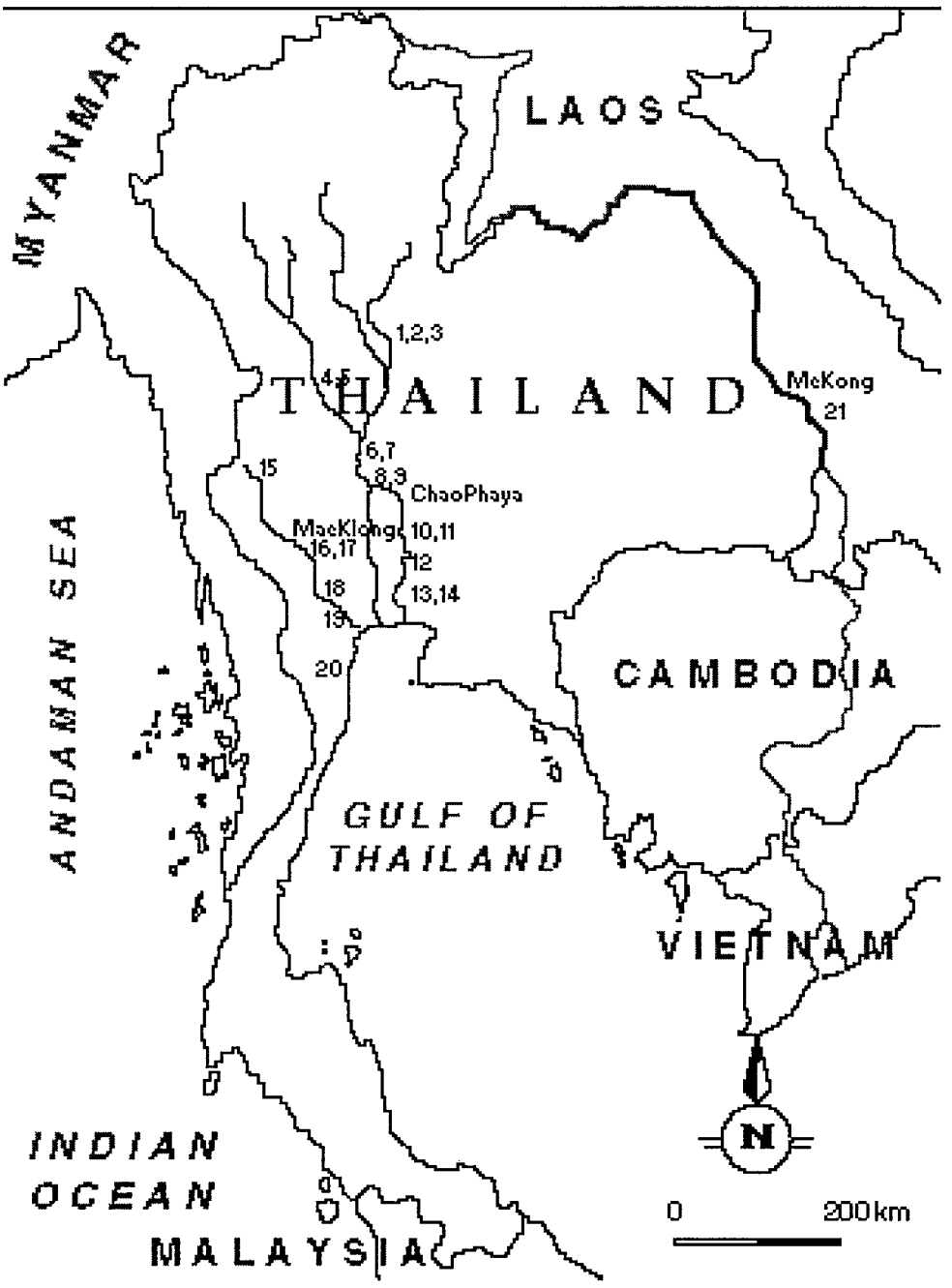


Figure 3.1 Sample sites of *Puntius gonionotus* in Thailand. Number refers to location in Table 3.1.

Figure 3.2 Allele size distribution for populations in the ChaoPhaya (Fig 3.2A-D) and the MaeKlong Rivers (Fig 3.2E-H).

Pgon-17 (ChaoPhaya River)

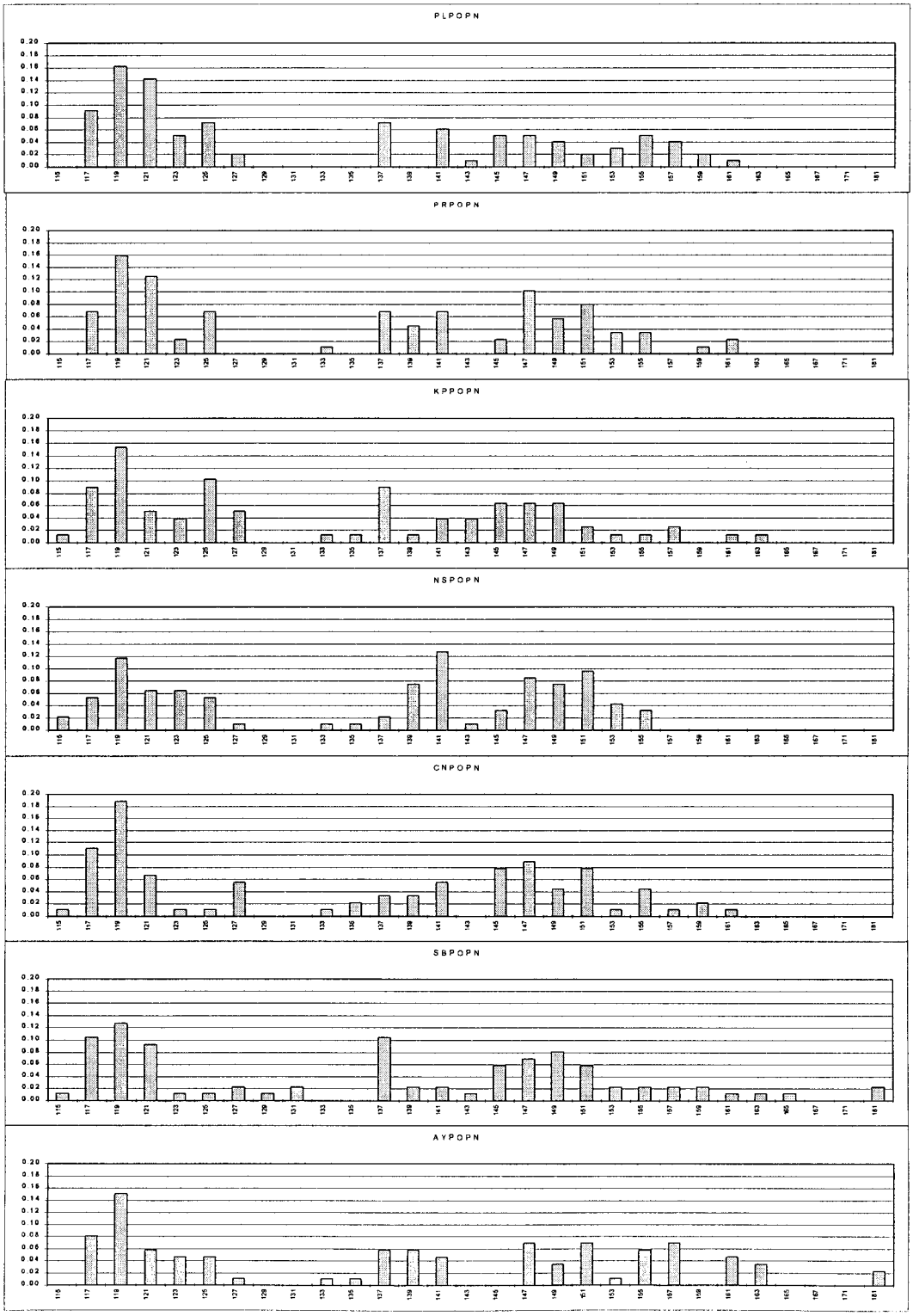


Figure 3.2 A

***Pgon-69* (ChaoPhaya River)**

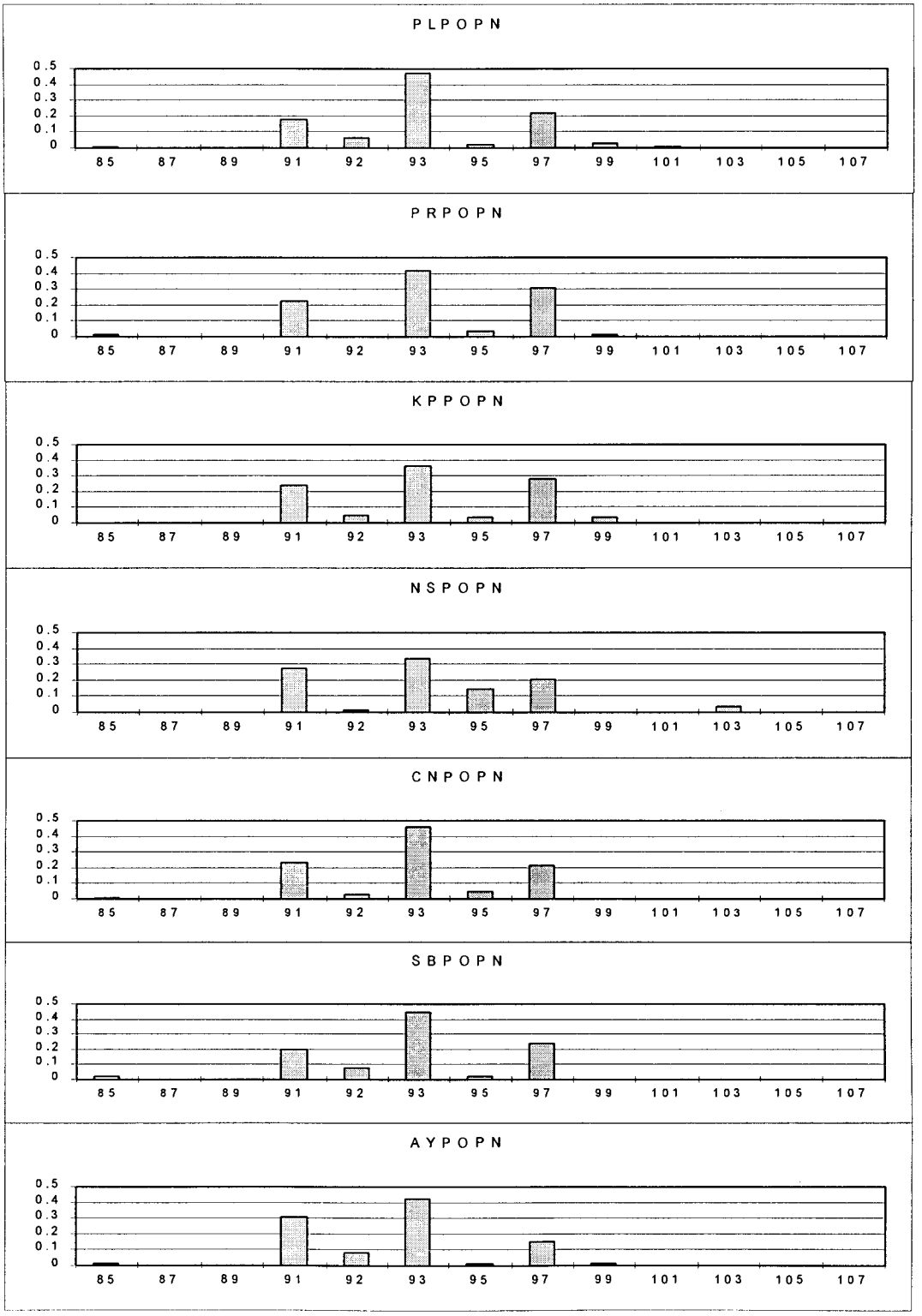


Figure 3.2B

Pgon-75 (ChaoPhaya River)

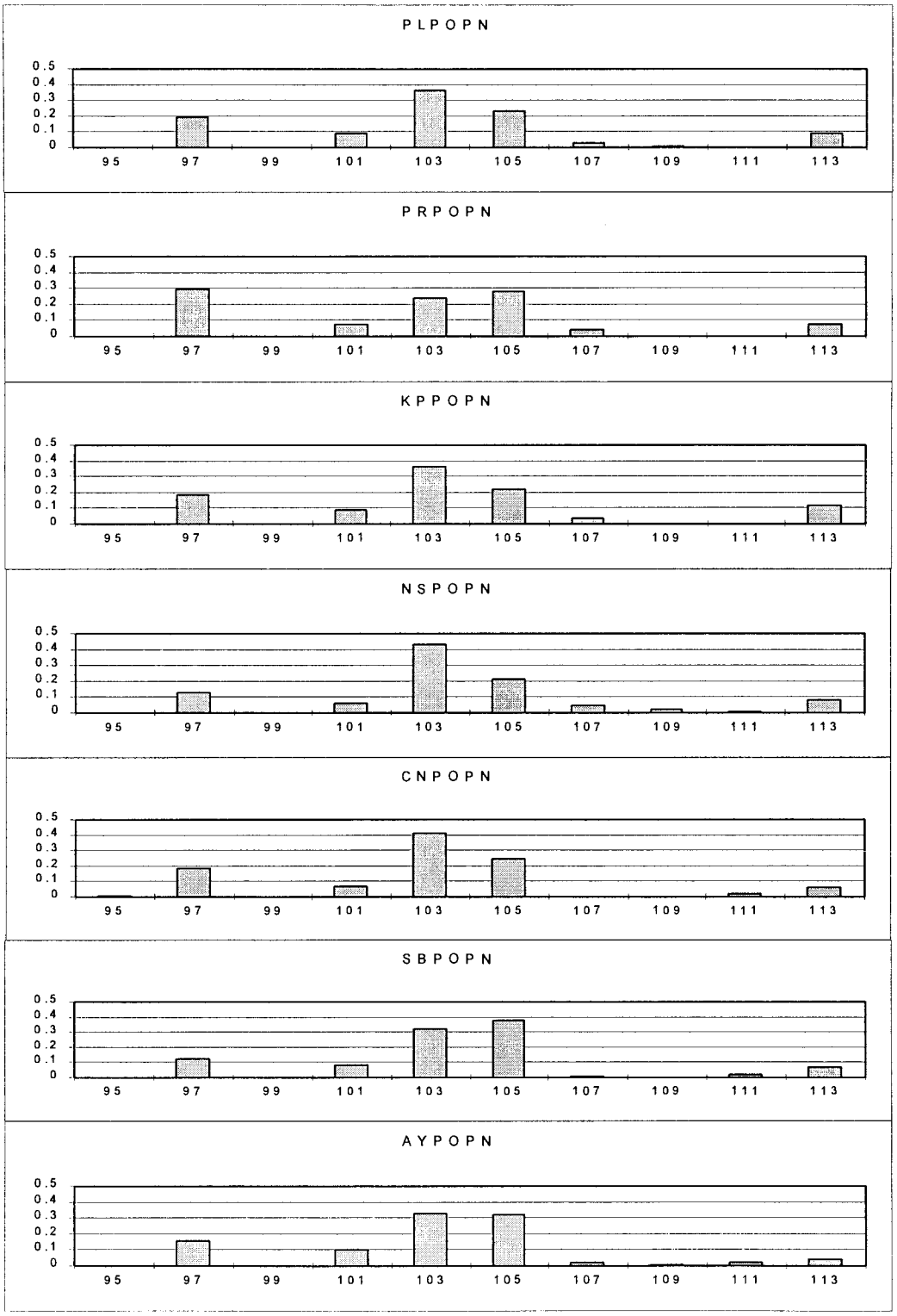


Figure 3.2C

Pgon-79 (ChaoPhaya River)

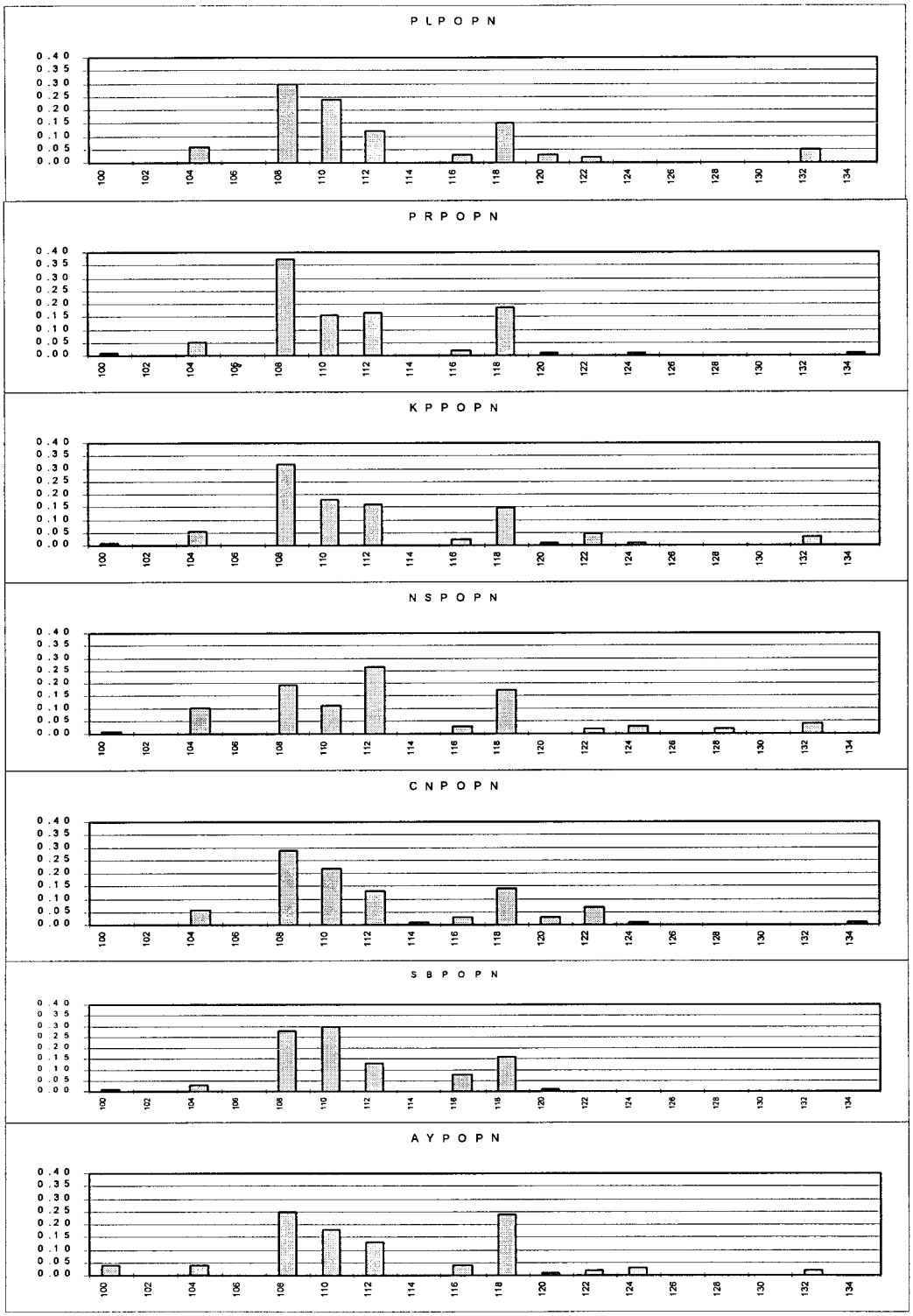


Figure 3.2D

***Pgon-17* (MaeKlong River)**

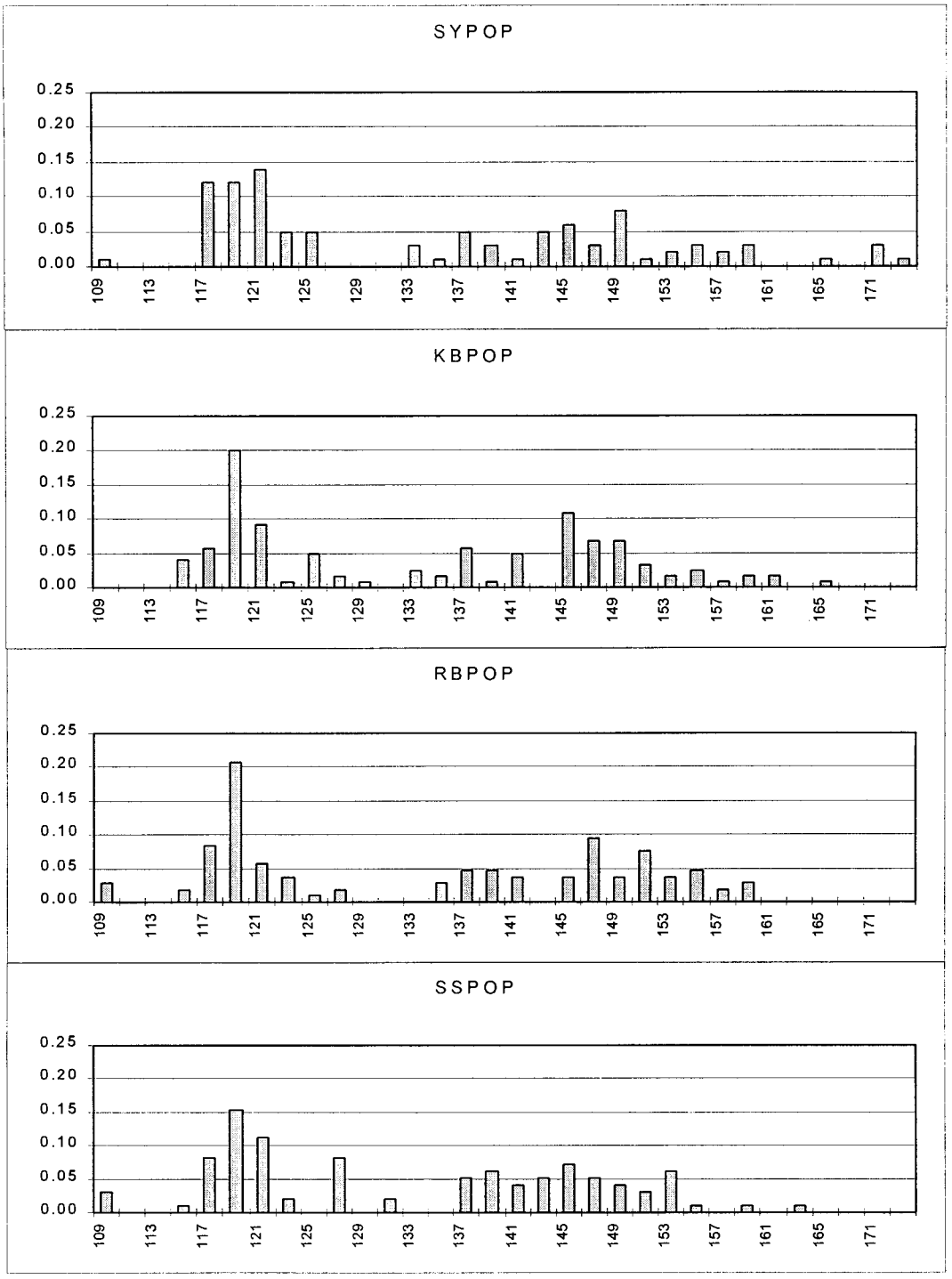


Figure 3.2E

***Pgon-69* (MaeKlong River)**

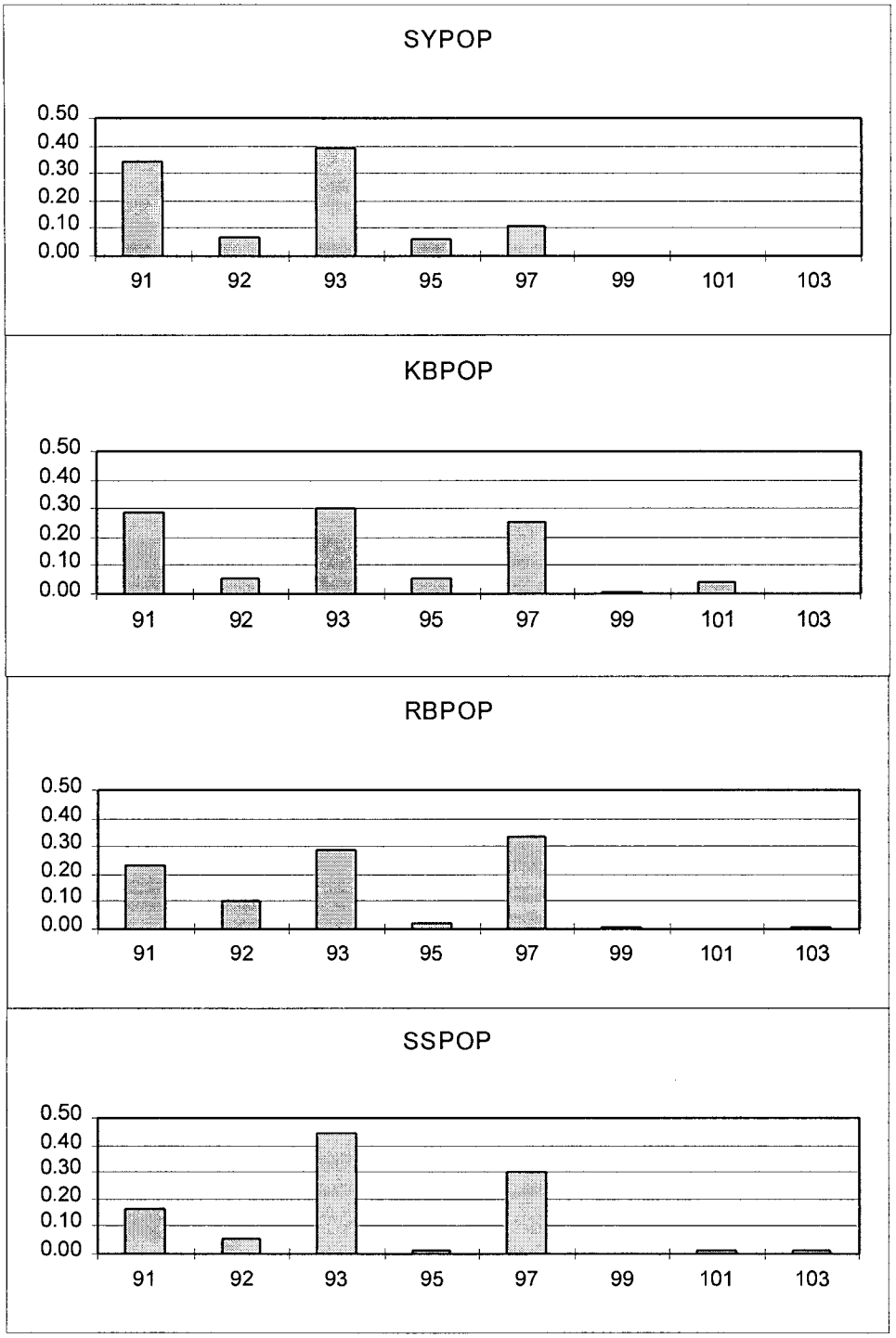


Figure 3.2F

***Pgon-75* (MaeKlong River)**

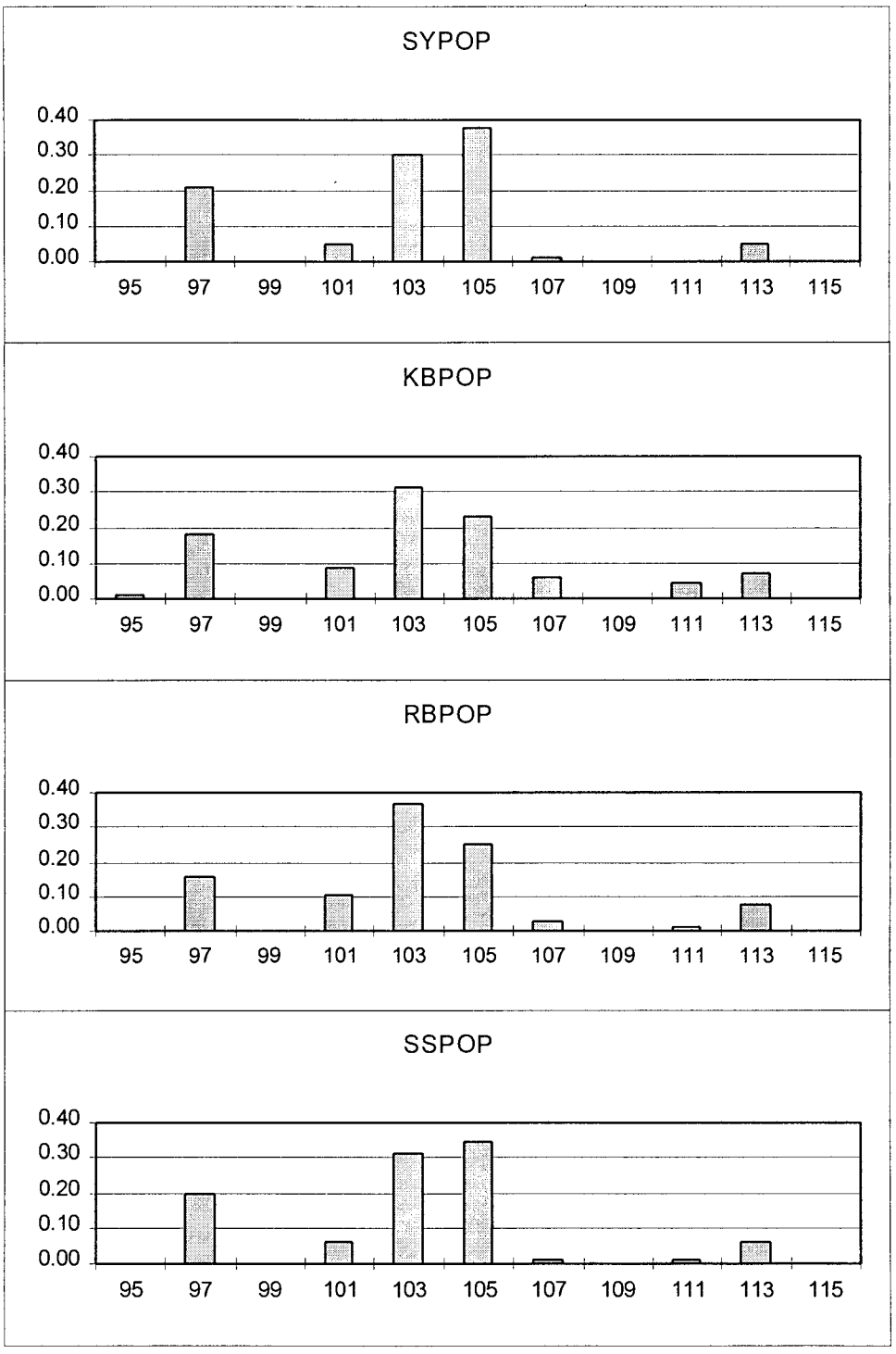


Figure 3.2G

***Pgon-79* (MaeKlong River)**

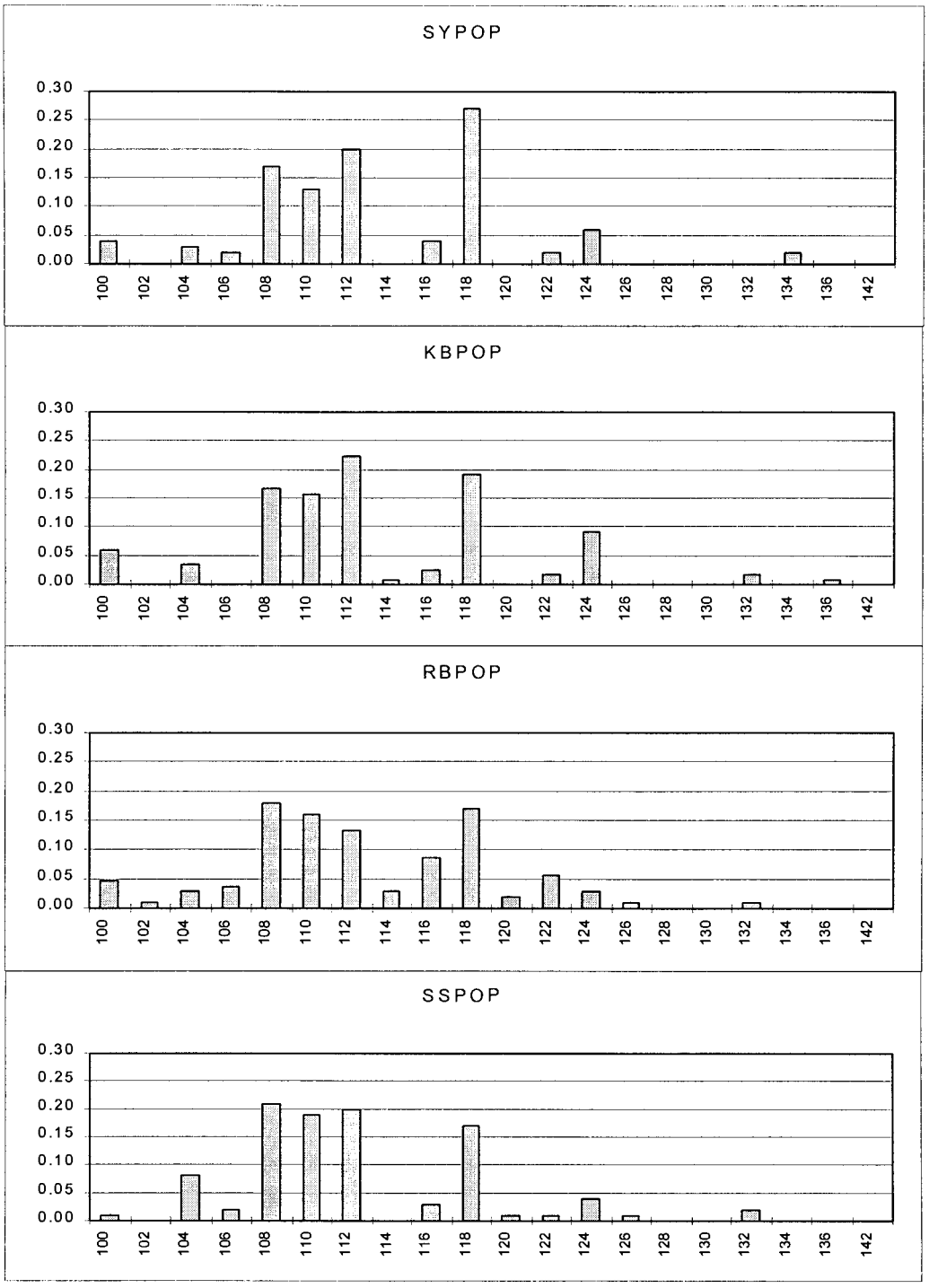


Figure 3.2H

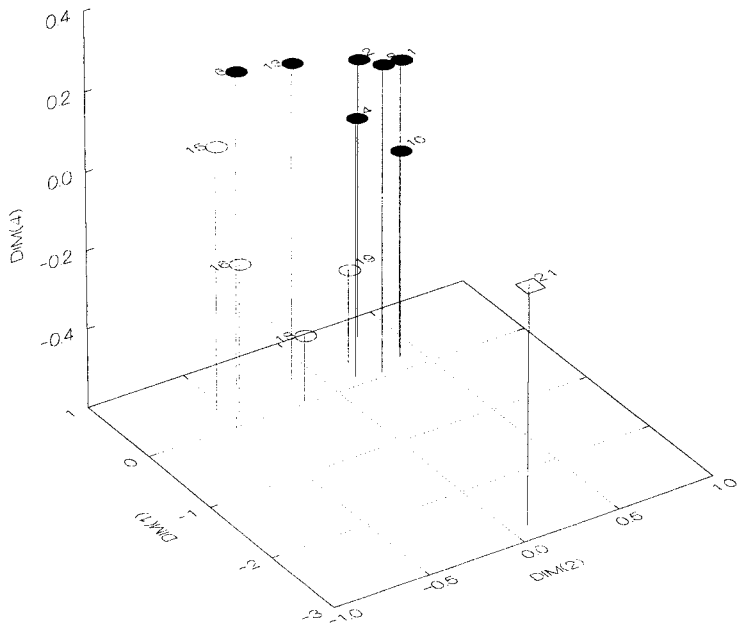


Figure 3.3 Genetic relationships among the studied populations of *Puntius gonionotus* in three rivers in Thailand. The plot is against dimension 1, 2 and 4 of the configuration produced by a multidimensional scaling (MDS) analysis of Modified Rogers' genetic distances. Closed circles indicate ChaoPhaya's populations, open circles indicate MaeKlong's populations, and an open square indicates MeKhong's population. Numbers refer to populations in Table 3.1.

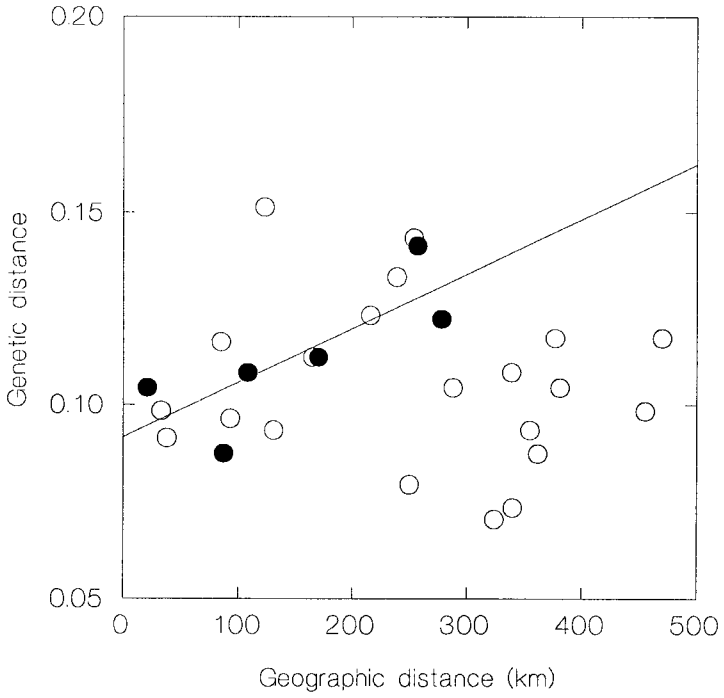


Figure 3.4 Relationship between genetic distance and geographic distance among *Puntius gonionotus* populations. Modified Rogers' genetic distance are plotted against geographic distances between all pairwise combination of populations within a river. Open circles indicate all pairwise combinations in the ChaoPhaya river, and closed circles indicate those of the MaeKlong river. A regression line ($Y = 0.092 + 1.42X\ 10^{-4}\ X$; $P=0.065$) is plotted for all points from MaeKlong pairs.

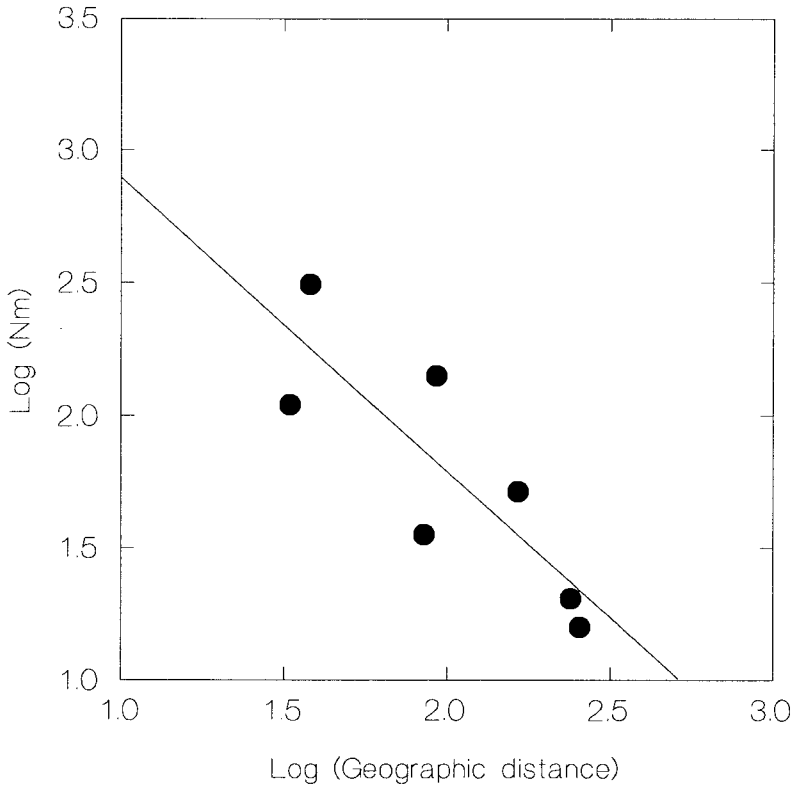
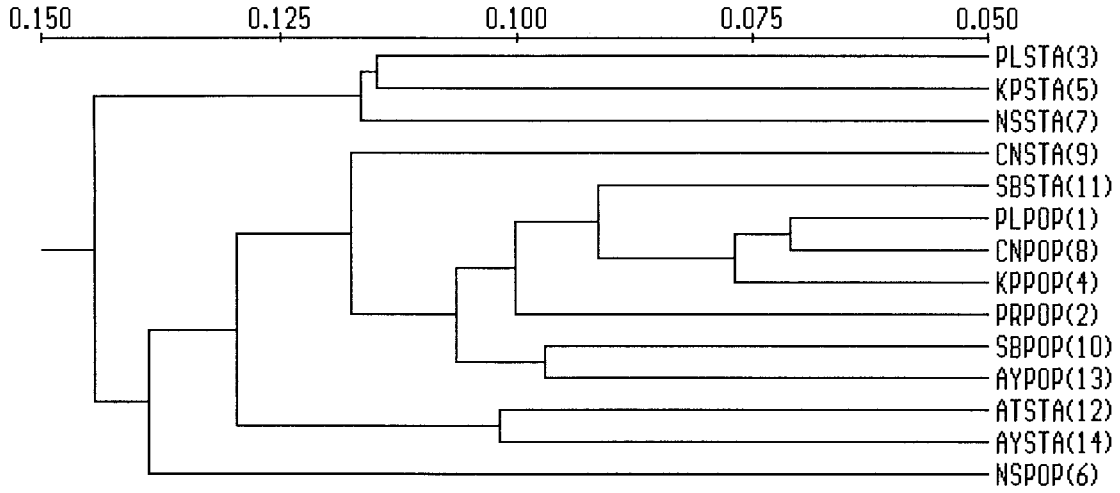
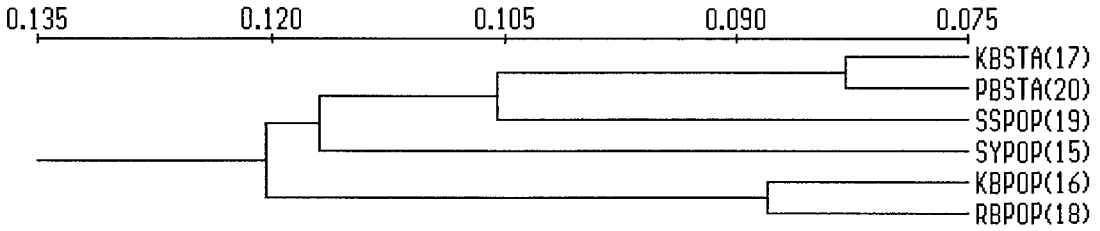


Figure 3.5 Isolation by distance (Slatkin, 1993): the log-log plot of gene flow [*Nm* = 1/4(1/F_{ST}-1)] versus geographic distances among the adjacent pairs of ChaoPhaya population, a least square regression line is *Y = 3.998 - 1.105X,* r^2*=0.705 P=0.018.*



(A) ChaoPhaya



(B) MaeKlong

Figure 3.6 UPGMA dendrograms clustering Rogers' modified genetic distance among populations of *Puntius gonionotus* in ChaoPhaya and MaeKlong regions.

Chapter 4

Spatially subdivided ***Puntius gonionotus*** *(Bleeker)* in Fisheries Stations: a potential use for genetic conservation

ABSTRACT

Fisheries stations represent the largest number of captive stocks of *Puntius gonionotus* (Bleeker) in Thailand. Large numbers of the fish are produced from the stations and used for restocking throughout the country. Samples from broodstock of 29 fisheries stations in two regions of Thailand were examined for genetic variation at four polymorphic microsatellite loci. Significant population differentiation was found among all 29 stocks (θ = 0.036), where the hatchery stocks in the north-east region exhibited significantly higher differentiation (θ = 0.0378) than stocks in the central region (θ = 0.0136). Average Cavalli-Sforza and Edwards (1967) chord distances between hatchery stocks within the regions were 0.273 and 0.334 in the central and north-east group, respectively. The average chord distances between stocks from different regions was 0.345. Nonmetric multidimensional scaling analysis of chord distances clearly differentiated most of the stations from the central and north-east groups. Evidence of reduction of genetic integrity between regions was found in stations whose stocks were transferred across the regions. Significant differences in allele frequencies were found on several occasions including between adjacent stations, and between stations where stocks were transferred or exchanged. Genetic drift is believed to be the major cause of the allelic differences. Hatchery stock management in Thailand is discussed from genetic point of view.

INTRODUCTION

Fish hatcheries are common in many developing countries where aquaculture has become an important source of protein for the national food consumption. Numerous fish of several species are produced, particularly from government operated hatcheries, for either aquaculture purposes or for release into natural waterbodies to enhance natural production. The two purposes are, however, potentially in conflict by their nature. For restocking programs, the desirable genetic goals for future survival of the species are to preserve genetic variation, avoid artificial selection and to maintain subpopulation structure. Aquaculture practices, on the other hand, which require selective breeding for improving the desired performance are likely to reduce the diversity of domestic stocks (Doyle *et al.*, 1991). The practice of stocking hatchery fish to enhance natural production has been employed for decades without such considerations. Awareness has grown in recent years concerning the incompatible nature of hatchery produced and wild fish, and this awareness has raised concerns that the use of hatchery fish in enhancement program may lead to declines, both productively and genetically, in many wild stocks of fishes (*e.g.*, Waples, 1991*a*; Stickney, 1994; Sheridan, 1995). General concerns have been building around the genetic diversity and integrity of the wild populations which may be reduced or altered by hatchery fish.

While all researchers have agreed in principle that genetic variation is a central component to biological conservation, the approaches to the problem are various. The most common recommendations are the use of local fishes for restocking and maintaining conditions in the hatchery similar to the environment to which the fish will face upon release (*e.g.*, Ryman, 1991; Stickney, 1994). Waples (1991*a*) suggested that

restocking program should not be considered unless there is adequate assurance of no genetic risks to the wild populations. Krueger *et. al.* (1981) suggested releasing numerous stocks in an unutilized habitat and allowing selection to filter the resulting genetic combinations, which may eventually produce strains that are well adapted to the local environment. Garcia-Martin *et.al.*(1991) recommended replacing the existing hatchery populations with local natural populations in their study on brown trout in Spain. In many situations, however, where natural populations have already been substantially contaminated by hatchery fish, such as the *Puntius gonionotus* populations of ChaoPhaya and MaeKlong rivers in Thailand (this study), Atlantic salmon populations in the Bothnia Sea, Finland (Koljonen, 1995), along the Norwegian coast (Lund *et. al.*, 1991) and South Western Iceland (Gudjonsson, 1991), and the brown trout populations in Lower Lough Erne, Northern Ireland (Taggart & Ferguson, 1986), replacing the existing hatchery stocks with the natural ones may not solve the problem. There is no doubt that the same situation would have occurred in many other populations where over-restocking of hatchery fish is being practiced. In such cases, the cultivated stocks may become a major component of the total diversity of the species due to the loss of natural gene pools.

Whether the fish are being produced for restocking or aquaculture, genetic diversity of the species is of interest. Fisheries biologists and conservationists have recognized the important of genetic diversity for future survival of the species while aquaculturists may recognize it as the source of their future economic survival. Owing to unpredictable changes in consumer preference, economic conditions and husbandry techniques, many have recommended the development of biologically divergent breeds as a protection against uncertainty (*e.g.*, Land, 1981; Smith, 1985). Doyle *et. al.* (1991)

proposed a selective diversification program through which genetic diversity of aquaculture stocks can be maintained by breeding programs, owing to genotype-environment interaction and farmer preferences. In their proposal, genetic diversity of aquaculture stocks should remain high at a metapopulation level that include all breeds. Smith (1985) suggested at least the order of 50 biologically divergent breeds would best serve the long-term economic interests of the agriculture community.

In Thailand, fisheries station stocks of *P. gonionotus* represent the largest number of captive stocks in the country and can be easily managed as a whole because they belong to a single organization, the Department of Fisheries (DOF) of the Thai Government. The Thai DOF was established in 1926 as a government agency concerned with living aquatic resources. In the early stages of the development of the DOF, interest was mainly given to freshwater fisheries owing to a great decline of natural production, especially in the central plain area. As the demands of food consumption increased, aquaculture has expanded rapidly throughout the country. To ensure success in development and management of both fisheries and aquaculture in the country, a number of provincial fisheries stations were established nationwide. These stations provide necessary technological know-how and appropriate management schemes for local fisheries and aquaculture development as well as providing fish fingerling for such activities. At present (1996), there are 87 fisheries stations, of which 56 are for fresh water, 23 are for brackish water, and 8 are for marine fisheries.

In this study, we investigated genetic diversity of the hatchery populations of *P. gonionotus* in many government fisheries stations in Thailand by the use of microsatellite DNA markers. The objectives were (1) to assess genetic variation within and among hatchery stocks, and then (2) to examine historical/cladistic relationships among stocks

so that the genetic structure of the hatchery stocks of *P. gonionotus* can be determined. The information obtained will provide the basis for recommendation for genetic stock management in fisheries stations and for conservation of genetic diversity of the species in Thailand.

MATERIALS AND METHODS

Studied populations

Puntius gonionotus samples were obtained from 29 provincial fisheries stations in Thailand during the period April 1994 to August 1994. There were 14 stations from the north and central region (central group) and 15 stations from the north-east region (north-east group) (Fig. 4.1 and Table 4.1). The central group covered the region lying from latitude 17°50' to 13° and from longitude 99°50' to 101°. Twelve stations (1-12) of this group are located on the ChaoPhaya River and its tributaries. Two other stations, i.e., station 13 and 14, are located on MaeKlong and Phetburi Rivers, respectively. The north-east group (15-29) covered a larger geographic area and extended from latitude 102° to 105° and from longitude 14°50' to 18°. The north-east region of Thailand consists of a saucer-shaped plateau which is flooded during the wet season and suffers from a scarcity of water during the dry season. The central region, on the other hand, is the comprehensive designation of the vast plain watered by the ChaoPhaya and its tributaries. Natural boundaries separating the north-eastern from the central regions are the Phetchabun Mountains and Dong Phya Yen Range.

A random sample of 50 fish (approximately 5% -10% of the total broodstock) was obtained from each station. Blood samples were collected and preserved in 100% ethanol for microsatellite DNA analysis.

Information on the broodstock, such as, its origin, the number of brood fish, fingerling production, etc. were obtained either from station records or from interviews with station personnel.

Microsatellite analyses

Samples of DNA extracted were subjected to PCR amplification using the primers developed from *P. gonionotus* genomic library. Four polymorphic microsatellites were surveyed, including *Pgon-17*, *Pgon-69*, *Pgon-75* and *Pgon-79*. DNA extraction and PCR procedures were previously described (see **Chapter 2**). Genotypes of individuals were scored relative to a sequencing ladder generated from single-stranded M-13 DNA (Yanisch-Perron *et al.*, 1985).

Statistical analyses

Genotype and allele frequencies at each locus were calculated for each population. Two tests were carried out to assess non-random associations between alleles at a locus (test of Hardy-Weinberg expectations) and between alleles at different loci (composite linkage disequilibrium test, Weir, 1990*a*). Probability of type-I error (*P*-value) for rejecting null hypothesis (H_0: samples are in HW equilibrium or population is in linkage equilibrium) were estimate using a Markov chain "approximate to exact test" technique followed the algorithm of Guo and Thompson (1992). Both tests were carried out using the GENEPOP computer program (Raymond & Rousset, 1995*a*). Combined

test for significance at all loci for each population using Fisher's combination of *P*-value method (Sokal & Rohlf, 1981). A sequential Bonferroni correction method was used to adjusted significant levels for multiple test (Hochberg, 1988; Lessios, 1992; Rice, 1989).

The genetic variability of the population in each station was assessed by calculating the mean number of alleles per locus, the proportion of individuals sampled that are heterozygous (i.e., direct-count heterozygosity, h_{obs}), the unbiased estimated of heterozygosity, h_{exp} (Nei, 1978) and the effective number of alleles per locus (Crow and Kimura, 1965). The first three variables were obtained from BIOSYS-1 (Swofford & Selander, 1989). Significant difference between h_{obs} and h_{exp} was tested using the independent sample *t*-test (Archie, 1985). In addition, fixation index of each population (F_{IS}) was calculated using Nei's (1973, 1977) method.

Heterogeneity of allele frequencies among the *Puntius* stocks within the DOF group (details in Table 4.1), between stations where stocks were exchanged (Fig. 4.2), and between geographically contiguous pairs based on a Gabriel network (Gabriel & Sokal, 1969) (Fig. 4.3) were determined by the Fisher test for R x C contingency tables (Raymond & Rousset, 1995*b*) and were obtained using the GENEPOP computer program. The Gabriel network defined populations as contiguous if the line connecting the two populations sites forms the diameter of a circle within which no other site lies. The probabilities of all loci were combined for an overall test using the Fisher's combination of *P*-value method (Sokal & Rohlf, 1981). The significant levels were adjusted for multiple tests using a sequential Bonferroni technique (Hochberg, 1988; Rice, 1989).

The population structure of the *P. gonionotus* stocks was analyzed by using Wright's *F*-statistic approach (Wright, 1951, 1978). F_{IT} and F_{IS} are the fixation indices of

individuals relative to the total population and its subpopulations, respectively. F_{ST} measures the amount of differentiation among subpopulations relative to the limiting amount under complete fixation. Values of ***F***, *f* and θ (unbiased estimate of F_{IT}, F_{IS} and F_{ST}, respectively; Weir & Cockerham, 1984) were calculated for each locus and their variances cited here are jackknifed estimates. The overall value for all loci was tested for significance (H_0: ***F*** or *f* or $\theta = 0$) by the bootstrapping method of Weir (1990*b*). The estimates were obtained from the computer program DIPLOIDX (modified from Weir, 1990*b*).

Cavalli-Sforza and Edwards (1967) chord distances were calculated between all pairs of populations using BIOSYS-1 computer program. The distance matrix was used to determine the pattern of genetic relationships among populations and was analyzed using the nonmetric multidimensional scaling (MDS) approach suggested by Lessa (1990) and carried out using SYSTAT (Wilkinson *et. al.*, 1992). The relationship between genetic and geographic distances was tested using a Mantel's test (Manly, 1994; Mantel, 1967) performed by the NTSYS-pc (Rohlf, 1993). Significance was determined using both an approximate *t*-test and a nonparametric test with 1000 random permutations of the matrices. A dendogram was constructed from the estimates Cavalli-Sforza and Edwards chord distances using the unweighted pair group method with arithmetic averages (*UPGMA*, Sneath and Sokal, 1973) to examine graphically the relative similarities among stations.

RESULTS

Broodstock information

Precise records of the origins of *P. gonionotus* hatchery stocks in Thailand do not exist. Information presented in this study is based on interviewed with station personnel. Partial details of origins and stock transfers, as far as they can be ascertained, are shown in Fig. 4.2. It is clear that hatchery stocks of the central group originated from the ChaoPhaya and its tributaries (TaChean Rivers) while stocks of the north-east group originated from natural populations in the northeast, such as MeKong, NamMun and NamChi Rivers. However, there were some stock transfers between both regions. Most stations obtained their stocks from other stations only once and thereafter replenished their stocks with the fish produced in the stations. Two stations (*NRSTA* and *UBSTA*) obtain part of their stocks from private farms (within the province) every year. The number of brooders varies from 300 to 8,000 fish.

All stations currently practice hormone-induced spawning to produce *Puntius* fingerlings. The procedures include hormone (pituitary extract, CG, or LH-RH analog) injection of female brooders to induce spawning followed by mass spawning in a hapa. The male to female ratio is 2:1. Each spawning batch consists of 15 to 100 females. Fertilized eggs are hatched in either water- or air-circulated ponds. The hatching period of *P. gonionotus* is 8 to 12 hours. Fry are fed with hard-boiled egg yolk for a few days before transfer to earthen pond for further nursing. Fingerling sizes of 2 to 5 cm are obtained after a month and are used either for aquaculture or restocking natural waterbodies. *Puntius* fingerling production takes place between March and September each year.

Non-random association tests

Results of the exact test for Hardy-Weinberg proportions are shown in Table 4.2. Single-locus tests of Hardy-Weinberg proportions indicated that only five out of 116 comparisons (4.31%) were significant deviant from the HW expectations ($P < 0.05$), however, all significances disappeared when a sequential Bonferroni correction for multiple testing was applied. Fisher's combined test over all loci indicated one out of 29 tests (3.45%) differed significantly from HW expectations but also lost its significance after sequential Bonferroni correction for multiple testing.

Composite genotypic linkage disequilibrium test results are shown in Table 4.3. After applying a sequential Bonferroni multiple test, linkage disequilibria were detected ($P < 0.05$) in seven tests (4.02%). The significant results included three stations of the central region (*AYSTA*, *SPSTA* and *UTSTA*), and two stations of the north-east region (*NKSTA* and *SNSTA*). The significances appeared to be random among pairs of loci tested and none of the stations broodstock exhibited linkage disequilibrium at all pairs of loci.

Genetic diversity within station stocks

Genetic diversity of each station stock is shown in Table 4.4. All station samples exhibited relatively high genetic diversity in all categories. The central group tended to have a higher average number of alleles per locus as well as effective number of alleles than the north-east group. The average number of alleles per locus ranged from 10.5 to 12.8 among the central stations and from 8.8 to 11.5 among the north-east stations. The effective number of alleles ranged from 4.829 to 8.152 among the central stations and

3.302 to 7.063 among the north-east stations. Mean observed heterozygosities were similar among all stations and ranged from 0.686 to 0.852. Eighteen stations samples had observed heterozygosities lower than expected. Only one stock (*RESTA*), however, showed a significant deficiency of heterozygotes in comparison with the HW expectations, which was about equal to that expected from a random occurrence (5% probability of a Type-I error). Fixation indices (F_{IS}) ranged from -0.0419 to 0.1016.

Genetic variation among station stocks

Allele frequencies in most of the DOF stations, particularly of the north-east group, were significantly different from each other (Table 4.5). In the central group, differences in allele frequencies were observed at three loci (*Pgon-17*, *75* and *79*) but none was observed at *Pgon-69*. The major difference in allele frequencies between stations within the central group occurred at locus *Pgon-79* (45% of the tests). The stations within the Karnchanaburi (4) and Ayutthaya subgroups (3) were very similar, where none and 15% of the tests were significant. Combined test (χ^2) for all loci indicated 17 pairs had allele frequency differences significant at the 0.05 level or lower and five pairs were not significant. In the north-east group, differences in allele frequencies were observed at all loci. The major differences occurred at locus *Pgon-17* in 25 of 31 tests (80%), although high percentages were observed at other loci (35% - 48%), were significant differences after a Bonferroni correction. Only three pairs (9%), all of which are in the Ubolratchathani subgroup, were not significantly different at all loci after adjusting *P*-value with a Bonferroni method. However, the combined tests (χ^2) for all loci indicated all pairs, except *RESTA-YSSTA*, had allele frequencies differences significant at 0.05 level or lower.

Significant differences in allele frequencies were also found in many of stations within contiguous groups (Table 4.6). Many contiguous pairs in the central group were relatively more similar than that of the north-east group. Five of 13 pairs (38%) in the central group were not significantly different while four of 15 pairs (27%) in the north-east group were not significantly different. Five and three pairs in the central and north-east group, respectively, were different at only one locus. All contiguous pairs were similar at locus *Pgon-69*, where none was detected in the central group and four were detected in the north-east group. The combined test (χ^2), however, indicated allelic frequencies differentiation for most of the contiguous pairs (78%).

Allele frequencies were compared between stations where stocks were known to have been exchanged or transferred (Table 4.7). Allele frequencies appeared to be very similar among transferring stations, where 13 pairs (52%) were not significantly different at all loci and only two pairs (*MSSTA-LBSTA* and *UDSTA-NPSTA*) were significantly different at all four loci after a Bonferroni correction. Overall, significant differences were dispersed among four loci, but the major differences occurred at the locus *Pgon-17*, where 11 of 25 tests (44%) were statistically different after Bonferroni correction. Combined tests (χ^2) for all loci indicated 20 pairs had allele frequencies significant at the level 0.05 or lower and only five pairs were not significantly differentiated. The differentiation between transferring stations did not appear to depend on the time of separation. It is evident, however, that all stations where stocks were transferred three to four years ago (*i.e.*, in 1991-1992) were very similar to each other.

Genetic structures of hatchery populations

Population structures of the *P. gonionotus* hatchery stocks were assessed using the hierarchical *F*-statistics method (Wright, 1951, 1978). The results of analysis indicated significant differentiation for *P. gonionotus* among all stations (Table 4.8) and therefore, suggested significant substructuring of the hatchery stocks in this species in Thailand. Across all 29 stations studied, on average 3.6% of the total variance of allele frequencies was due to genetic differences between stocks (mean θ = 0.0360). More than 90% of the total genetic diversity was found within any given stocks. The average θ among the stations in the central group was 0.0136 (95%CI: 0.0085 to 0.0172) which was significantly lower than that of the north-east group (θ = 0.0378; 95%CI: 0.0220 to 0.0542), suggesting significantly higher differentiation among the north-east stocks than among the central stocks. Significant positive values of ***F*** (F_{IT}) were observed at all loci when calculated across all stocks. Their positive but low values suggested slightly lower number of heterozygous individuals relative to that expected when the data were pooled for all stocks. Heterozygous deficiencies relative to the HW expectations are predicted for a substructured population by the Wahlund effect (Wahlund, 1928). Significant positive values *f* (F_{IS}) were observed at locus *Pgon-75* (*f* = 0.0366) and *Pgon-17* (*f* = 0.0312) among the central stations and the north-east stations, respectively, and thus indicating that there was, on average, a deficiency of heterozygous individuals within each stock at these loci.

The population structure of *P. gonionotus* hatchery stocks was revealed graphically by a nonmetric multidimensional scaling (*MDS*) method (Fig. 4.4). *MDS* is designed to depict a complex set of relationships among samples, initially represented by a matrix of pairwise distances or similarities, in a space of a few dimensions without any

significant loss of resolution (Lessa, 1990; Manly, 1994). The *MDS* method is preferable to other hierarchical clustering methods, such as *UPGMA*, neighbor-joining, etc. in revealing overall structure of population where a certain degree of genetic exchange between population exists or non-linearity among populations is expected. Most hatchery stocks, including in this study, are established from several pre-existing sources to create broader diversity of the stocks, and therefore exhibit a so-called reticular pattern (Lessa, 1990) rather than linear pattern among stocks. A three-dimensional solution analyzed on the pairwise Cavalli-Sforza Edward chord distance matrix of all *P. gonionotus* stocks (Table 4.9) was used and its corresponding stress value was 0.0917 suggesting a "good" goodness of fit between configuration distances and the disparities (Rohlf, 1993). The plot of dimensions 1 and 2 indicated a separation of the central group stations from most of the north-east group stations (Fig. 4.4). The central group were relatively similar and tended to cluster in one group. The north-east group, on the other hand, were largely differentiated and scattered into three clusters, one of which (consisted of *BRSTA*, *KSSTA* and *UBSTA*) overlapped into the central cluster.

Average Cavalli-Sforza and Edwards (1967) chord distances between hatchery stocks of *P. gonionotus* within the central group and the north-east group were 0.273 and 0.334, respectively. The average chord distances between hatchery stocks from different groups was 0.345. The large chord distance among hatchery stocks in the north-east stations was in agreement with the *MDS* analysis which indicated the stocks were highly divergent from one another.

To examine the effect of geographical distance on genetic relationships among hatchery stocks of *P. gonionotus* in Thailand, pairwise geographical distances were calculated and compared against pairwise Cavalli-Sforza and Edwards chord distances

(Table 4.9). A Mantel's test of this relationship suggested a relatively poor relationship between genetic and geographical distances with a normalized Mantel statistic, *r*, of 0.482. The randomization test indicated a significant positive correlation between the two distance matrices, out of 1,000 random permutations none were equal to or larger that the observed Z-value and the one-tail probability of [random Z ≥ observed Z] was 0.0010 (Manley, 1994). This is consistent with the probability estimated from the approximate *t*-test(t = 7.829 and p[random Z < observed Z] = 1.000) (Rohlf, 1993).

DISCUSSION

Breeding structure and genetic diversity in station stocks

The genotypes of the progeny are determined by the union of the parental gametes to form zygotes. This union of gametes is influenced by the type of mating of the parents. Therefore, the genotype frequencies in the offspring generation are directly influenced by genotypes of the mating pairs in the parent generation. Current practice in Thailand is to produce *P. gonionotus* by hormone induction and natural mass spawning, where 15 to 100 females and 30 to 200 males are used. All females are selected according to their readiness to spawn and no intentional selection is practiced. One of the major concerns in hatchery fish is inbreeding, as it has deleterious consequences, such as reduction of fitness and genetic diversity of the populations. Inbreeding is the mating of individuals with related and therefore similar genotypes and, therefore, increases homozygosity in a population. Small effective size of breeding stocks and overlapping generations in the stock promote inbreeding effects, even when mating is

random. Excessive homozygotes relative to binomial (Hardy-Weinberg) expectations may be detected statistically by either a test for HW proportions or linkage (gametic) disequilibrium. Departures from Hardy-Weinberg expectations also indicates a population undergoes either selection (if locus is under selection or closely linked with a selected locus), non-random mating, inbreeding or recent mixing of populations. Similarly, departure from gametic disequilibrium may be caused by selection, lack of recombination (*i.e.*, physical linkage), genetic drift and population subdivision (Waples & Smouse, 1990). The properties and limitations of the tests has been discussed previously (see **Chapter 3**).

Despite information obtained from hatchery personnel indicating potential departures from HW expectations such as recent mixing of preexisting stock, and mixing broods from many generations, none of the samples showed a significant departure from the HW expectations. A few tests, however, indicated significant linkage disequilibrium which, unlike HW disequilibrium, persists for several generations. Failing to detect discrepancy from HW expectations was, in part, due to the statistical power of the test. The HW test, for example, is a procedure prone to Type-II error in detecting statistically significant effects of inbreeding (Ward & Sing, 1970; Haber, 1980). Therefore, it has been suggested that "...little can be made of the non-significant results, except in comparison with significant ones" (Lessios, 1992).

The non-significant results in this study indicated no evidence of strong levels of selection or assortative mating in the fisheries stations broodstock. My past experience in fisheries stations supports this finding. The small facilities and the vast amount of fingerlings produced (each station produces approximately 2-5 million of *P. gonionotus* fingerlings a year) limits selection practice. Significant linkage disequilibrium detected in

five stations may be the result of either recent mixing of several gene pools and/or small effective number of breeders in comparison to other stations. Although several stations whose records indicated mixed sources, such as *PLSTA*, *ATSTA*, *LBSTA*, *etc.*, did not exhibit disequilibrium. Slatkin (1994) demonstrated that the chance of detecting linkage disequilibrium was very low, even in completely linked neutral loci, in a rapidly growing population. The power of detecting significant linkage disequilibrium in a mixed population is also reduced if genetic distances between source populations are small or an unequal mixture where one population contributes only small proportions (Waples & Smouse, 1990). Without precise records of stock history, it would be difficult to determine either the cause of discrepancy (or non-discrepancy) from the equilibrium or non-detectable disequilibrium.

All station broodstocks appeared to have relatively high genetic diversity based on microsatellite DNA variation. The values were similar to those reported in the natural populations (see Chapter 3). Most fisheries stations maintain relatively large number of broodstocks and the breeders are normally replenished from collections of fish produced throughout the year, which probably helps maintain the high genetic diversities of the stocks. Generally, the northeast group had a slightly lower number of alleles per locus and effective number of alleles than the central group. Based on the information available, the station stocks originated, either directly or indirectly, from the natural populations within the region. The previous study (Chapter 3) indicated that natural population in the MeKong (northeast region) had a lower genetic diversity than the natural populations in ChaoPhaya and MaeKlong Rivers. There was no indication of significant difference in broodstock management between the stations of the two regions.

Population structure of hatchery fish

Hatchery stocks of *P. gonionotus* in Thailand appear to exhibit a significant substructuring of genetic variation (θ = 0.036) where the central group of stations were more homogeneous (θ = 0.0136) and the north-east stations were largely heterogeneous (θ = 0.0378). The analysis of genetic distance within and between regions, and the *MDS* analysis supported this evidence. The global genetic relationships of the *Puntius* hatchery stocks as revealed by the *MDS* analysis (Fig. 4.4) indicated three distinct groups of stations. Two distinct groups consisted of only stations in the north-east region while the third one consisted of all central group stations and three of the north-east group (*BRSTA*, *NRSTA* and *UBSTA*). The genetic discreteness of hatchery stocks appeared to be more consistent with the known history of their origins rather than geographic distribution. In fact, the relationship between geographical and genetic distances among the station stocks was only moderate (normalized Mantel's statistic, r = 0.482). The finding suggested that the stock transfer among fisheries stations were less dependent on the proximity of the stations. Available information about stock transfer also confirmed that stocks were likely to be exchanged among or obtained from stations whose station managers have good relations.

The original sources of the hatchery stocks of *Puntius* in Thailand are assumed, also by interview information, to be from natural populations within the region. This probably caused the strong differentiation between the regions. Thereafter, the genetic differentiation among hatchery stocks appeared to be largely maintained (or reduced) by management practices such as stock transfer. The evidence for this can be clearly seen from the *MDS* plot (Fig. 4.4) where the stocks, *UTSTA*(3), *NSSTA*(4) and *KPSTA*(6), were placed in the middle between the central and north-east groups. All three stations

stocks were, in part, transferred either directly or indirectly from *KSSTA*. The subgroup *KKSTA*(15)-*NKSTA*(21)-*SKSTA*(27)-*SRSTA*(28) stocks were also related. There was no precise information on stocks *BRSTA*(16), *NRSTA*(19) and *UBSTA*(24), but the *MDS* plot suggested part of their stocks may have originated from the central origins. Information obtained from stations record indicated some proportion of their stocks were from private fish farms, which probably purchased *Puntius* fingerlings from hatcheries in the central region. Contingency tests of differentiation between transfer stocks also indicated significances in several occasions, even in the most recent transfer such as *KSSTA-NSSTA* and *UDSTA-NPSTA* in 1993 and 1992, respectively. It is expected, however, that such differences might be largely due to random drift as either a small number of broods or fish from a small group of parents were transferred.

It is difficult to distinguish the role of selective and non-selective forces in genetic differentiation. It has been suggested, however, that if the gene frequency variation is homogeneous over loci, then it is most likely to be the result of random processes as they affect all loci simultaneously and to the same extent (Lewontin & Krakauer, 1973; Schaal, 1975; Varvio-Aho, 1983). The estimate F_{ST} (θ) values for individuals loci ranged from 0.0058 to 0.0181 and from 0.0173 to 0.0623 among the central and north-east groups, respectively. The values were not significantly different from each other except that of *Pgon-69* in the central group. Therefore, it seems likely that the genetic constitution of *P. gonionotus* in both regions in Thailand can be accounted for, to the large extent, by random processes.

Broodstock management and conservation of population diversity

Similar to other captive populations, hatchery stocks are considered finite populations and, therefore, are vulnerable to genetic drift and inbreeding, particularly when the effective sizes are small. Genetic drift in a small population may lead to the loss of alleles, even favorable ones, and diminishes the potential to adapt to a changing environment (Vrijenhoek, 1985). Inbreeding, on the other hand, results in increased levels of homozygosity and may expose deleterious effects of the genes. These may lead to reduced fitness of individuals in a population and, thus, increase the chance of extinction of the populations. Therefore, it is essential that hatchery stocks must be carefully monitored and properly managed to reduce such effects.

The effective population size (N_e) is an important parameter influencing genetic structure of small populations. It has been suggested that an effective population size (N_e) of about 50 will be sufficient to keep inbreeding effect to a minimum in a population without great concern (Frankel & Soulé, 1981). However, if the goal is also to maintain genetic variation of a population so that its long-term adaptive potential is retained then the minimum N_e of 500 are suggested (Franklin, 1980). The N_e estimates for all Thai hatchery stocks using data on linkage disequilibrium (Hill, 1981; Waples, 1991*b*) were negative (data not shown). The formula for N_e estimate from unlinked loci is (Waples, 1991*b*):

$$N_e = 1/[3 \times (r^2 - 1/S)],$$

where S is the sample size and r is the correlation among alleles and is calculated from

$$r = \frac{D}{\sqrt{p(1-p)q(1-q)}}$$

where D is Burrow's composite measure of disequilibrium (Cockerham & Weir, 1977), and p and q are frequencies of allele A at locus 1 and allele B at locus 2, respectively. The formulae indicated that negative estimates of N_e is possible when $r^2 < 1/S$. The situation can arise by, in theory, sampling error in a population in which the true N_e is large and linkage therefore low ($r^2 \rightarrow 0$). Sample size is also important. The harmonic mean of the S in N_e equation indicates small sample sizes are likely to yield an uninformative negative estimate of N_e. The sample sizes of around 100 fish and more than 6 loci should produce a realistic estimate of N_e (Bartley *et al.*, 1992; Waples, 1991*b*).

The negative N_e estimates in this study may be attributed mainly to small samples (n=50). The information obtained from station personnel indicated that most stations maintain relatively large number of brooders and the fact that they were replenished from several spawning groups should guarantee relatively large N_e. This was supported by the relatively high genetic diversity found within all hatchery stocks.

Stock transfers can result in either reduction of alleles number (due to sampling error) or renewing genetic variation in a population. Reduction of the average and/or effective number of alleles per locus was observed in several occasions when stocks were transferred, for example among the transferred group NK(21) → KK(15) → KS(17) → NP(22), average alleles per locus were 10.5, 10.0, 9.0, 8.8, respectively (Fig. 4.2). An increase in the average and/or effective number of alleles per locus were also found, particularly when two or more sources were mixed to establish new stocks, for example,

among group AY(7), SP(12) and AT(8) and group MS(18), SP(12) and LB(10). These changes are random processes and thus depend upon the effective number of fish being transferred, the number of mixing stocks, and genetic differences of the stocks establishing the new stock. Unfortunately, precise records on stock transfer are not available for analyzing such effects quantitatively.

Maintaining the genetic integrity of populations is also an important issue in genetic conservation. The *Puntius* hatchery stocks in Thailand have a clearly established population substructure related to their geographic distributions. The genetic differentiation between the central and north-east groups appeared to be due to their different primary origins. The geographic and genetic relationships among hatchery stocks in this study was relatively low (normalized Mantel's statistic, $r = 0.482$) suggesting stock transfer was less dependent on geographic proximity. Many of the transfers, as obtained from station personnel, were socially influenced regardless of geographic distances between stations. Such practices pose a very high risk to the genetic integrity between regions if station managers have been transferred across regions. It is of importance, therefore, that the DOF restrict its policy about stock transfers or exchange. Evidence of reduction in genetic integrity was observed in many stations where stocks were transferred across regions (Fig. 4.4). In these cases, replacing the existing stocks with the one from the region is recommended.

Recommendations

The general goals of managing hatchery stocks should be to avoid inbreeding depression and to maintain genetic variation both within and between stocks. By maintaining a large effective number of breeding individuals or effective population size

(N_e), the effect of inbreeding can be minimized. N_e is a function of the harmonic mean broodstock number and therefore the smallest population will contribute the most effect. An unequal sex ratio or reduced numbers of population size in successive generations result in decreased N_e (Falconer, 1981). The current practice in the *Puntius* breeding program in Thailand where an unequal sex ratio is used (1 female: 2 males) undoubtedly decreases N_e. In addition, not all males will have the opportunity to breed, since large numbers of fish are put into the same breeding unit and the *Puntius* breeding behavior is competitive in nature. This should further reduce N_e. It is, therefore, recommended to, whenever possible, use an equal sex ratio in breeding. Artificial insemination, where eggs and milt are stripped and mixed together in a container (1 female and 1 male), is the best approach in controlling sex ratio. This practice has been abandoned by many hatcheries due to its labor intensive and time consuming nature. Maintaining equal number of N_e for each sex in successive generations may be difficult to achieve as to physically tagging at fry stage is not possible. Genetic markers, such as microsatellites can be used to identify individual relationships (Herbinger *et al.*, 1995) but the cost may be too great for routine farm management.

The deleterious effects of an erosion of genetic variation can be reduced by gene flow renewing the genetic variation in a population (Van Treuren *et. al.*, 1991). This may be used as a management tool to counteract the effect of inbreeding depression or allelic loss. The *MDS* analysis of genetic distances between hatchery stocks indicated that if stocks were to be transferred within the DOF administration groups (refer to Table 4.1), it would likely result in the reduction of genetic integrity of the whole population. Figure 4.5 shows that many of the stations within the same DOF group are genetically distant. In this case, cluster analysis (*UPGMA*) on genetic distances provides a convenient

graphical display of genetic similarity (or differences) among populations and can be used as a guideline for stock transfer. The *UPGMA* dendogram of hatchery stock of *P. gonionotus* in Thailand is shown in Figure 4.6. Stock transfer should be done within genetic proximity groups so that the overall population substructure, and thus genetic diversity, can be maintained.

Finally, hatchery managers should start keeping quantitative records, such as number of breeders, replacement, and stock transfer *etc.*, of their stocks. Such information will increase the efficiency and accuracy of analysis on the genetic "health" of their stocks.

Table 4.1 *Puntius gonionotus* sample collections. Grouping of stations are acccording to the DOF, Freshwater Fisheries Division network operation.

Group	Fisheries Station	Abbrevi-ation	Station	Broodstock size
Central group				
1. Phitsanulok	Phitsanulok	*PLSTA*	1	Female: 1,200 Male: 1,800
	Tak	*TKSTA*	2	Female: 1,000 Male: 1,000
	Uttaradit	*UTSTA*	3	Female: 2,400 Male: 1,000
2. Nakhonsawan	Nakhonsawan	*NSSTA*	4	Female: 800 Male: 900
	Kamphangphet	*KPSTA*	5	Female: 700 Male 500
	Phichit	*PCSTA*	6	Female: 3,000 Male: 4,000
3. Ayutthaya	Ayutthaya	*AYSTA*	7	Female: 2,000 Male: 2,000
	Angthong	*ATSTA*	8	Female: 700 Male: 800
	Chainat	*CNSTA*	9	Total: 2,000 - 3,000
	Lopburi	*LBSTA*	10	Female: 700 Male: 200
	Singburi	*SBSTA*	11	Total: 300 - 400
	Suphanburi	*SPSTA*	12	N/A
4. Karnchanaburi	Karnchanaburi	*KBSTA*	13	Female: 800 Male: 600
	Phetburi	*PBSTA*	14	Female: 1,000 Male: 500
North-east group				
5. Khonkaen	Khonkaen	*KKSTA*	15	N/A
	Burirum	*BRSTA*	16	N/A
	Kalasin	*KSSTA*	17	Female: 1,200 Male: 1,800
	Mahasarakram	*MSSTA*	18	Female: 400 Male: 300
	Nakhonratchasrima	*NRSTA*	19	N/A
6. Udornthani	Udornthani	*UDSTA*	20	Female: 400 Male: 400
	Nongkhai	*NKSTA*	21	Female: 300 Male: 200
	Nakhonphanom	*NPSTA*	22	Female: 600 Male: 400
	Sakonnakhon	*SNSTA*	23	Female: 5,000 Male: 3,000
7. Ubonratchathani	Ubonratchathani	*UBSTA*	24	Female: 400 Male: 600
	Mukdahan	*MDSTA*	25	Total: 5,000 - 6,000
	Roiet	*RESTA*	26	Female: 1,500 Male: 1,000
	Srisaket	*SKSTA*	27	Female: 3,000 Male: 1,000
	Surin	*SRSTA*	28	Total: 1,000 - 2,000
	Yasothon	*YSSTA*	29	Female: 750 Male: 1,400

N/A = information not available

Table 4.2 Results of the exact test of Hardy-Wienberg proportions at four microsatellite loci. Probabilities (*P-value*) were estimated using a Markov chain method (Guo & Thompson, 1992). Chi-square was a Fisher's combination of *P*-value method (Sokal & Rohlf, 1981). Significant levels were adjusted using a sequential Bonferroni technique.

Population	*Pgon-17*	*Pgon-69*	*Pgon-75*	*Pgon-79*	χ^2	*P*-value
Central group						
1.PLSTA	0.2474	0.0459	0.1336	0.8778	13.2	0.1038
1.TKSTA	0.8297	0.1896	0.7969	0.0896	9.0	0.3442
1.UTSTA	0.5059	0.8803	0.2939	0.4520	5.7	0.6858
2.NSSTA	0.0300	0.7386	0.5146	0.2911	11.4	0.1791
2.KPSTA	0.1061	0.1548	0.0939	0.5581	14.1	0.0788
2.PCSTA	0.2679	0.3793	0.6267	0.5144	6.8	0.5543
3.AYSTA	0.0910	0.7265	0.4418	0.2021	10.3	0.2469
3.ATSTA	0.7597	0.4162	0.9472	0.0969	7.1	0.5281
3.CNSTA	0.5682	0.6086	0.3563	0.2718	6.8	0.5591
3.LBSTA	0.7392	0.3522	0.1327	0.5473	7.9	0.4397
3.SBSTA	0.9215	0.5175	0.9847	0.3943	3.4	0.9088
3.SPSTA	0.6801	0.5850	0.0726	0.9301	7.2	0.5116
4.KBSTA	0.9948	0.7737	0.0158	0.6169	9.8	0.2805
4.PBSTA	0.8129	0.0413	0.1385	0.3624	12.8	0.1199
North-east group						
5.KKSTA	0.0995	0.5139	0.1696	0.6223	10.4	0.2353
5.BRSTA	0.4287	0.6934	0.3313	0.2104	7.8	0.4579
5.KSSTA	0.3998	0.2217	0.5907	0.9773	5.9	0.6533
5.MSSTA	0.6449	0.5917	0.2282	0.4565	6.5	0.5969
5.NRSTA	0.9467	0.7296	0.6499	0.7510	2.2	0.9752
6.UDSTA	0.0452	0.3033	0.8337	0.1433	12.8	0.1179
6.NKSTA	0.6310	0.8781	0.1618	0.2866	7.3	0.5022
6.NPSTA	0.4032	0.8236	0.0537	0.3226	10.3	0.2436
6.SNSTA	0.3103	0.9747	0.1436	0.7185	6.9	0.5438
7.UBSTA	0.0568	0.3996	0.1116	0.1178	16.2	0.0391
7.MDSTA	0.0794	0.9283	0.0625	0.7814	11.3	0.1877
7.RESTA	0.2951	0.3600	0.1240	0.5039	10.0	0.2630
7.SKSTA	0.4395	0.9506	0.3482	0.2734	6.4	0.5971
7.SRSTA	0.2139	0.9621	0.0267	0.7876	10.9	0.2083
7.YSSTA	0.2804	0.2759	0.2742	0.1798	11.1	0.1940

Table 4.3 Composite genotypic linkage disequilibrium analysis for all pair of loci in each population. The probabilities of type-I error (*P*-value) were estimated using a Markov chain technique (Guo & Thompson, 1992). Significant levels were adjusted using a sequential Bonferroni method (Hochberg, 1988; Rice, 1989).

Group	Population	*Pgon17-69*	*Pgon17-75*	*Pgon17-79*	*Pgon69-75*	*Pgon69-79*	*Pgon75-79*
Central region							
Phitsanulok	1. *PLSTA*	0.41620	1.00000	1.00000	0.09927	1.00000	0.71324
	2. *TKSTA*	0.37855	0.89428	0.93233	0.50647	0.64257	0.62861
	3. *UTSTA*	0.44660	0.31790	1.00000	0.21096	0.00137*	0.00719*
Nakornsawan	4. *NSSTA*	0.23788	0.53668	0.52757	0.47750	0.75215	0.13223
	5. *KPSTA*	0.16703	0.51053	0.10373	0.23818	0.50058	0.21815
	6. *PCSTA*	0.39379	1.00000	1.00000	0.28349	0.98955	0.27151
Ayutthaya	7. *AYSTA*	0.00784*	1.00000	0.49887	0.00836*	0.28579	0.03385
	8. *ATSTA*	0.44182	1.00000	1.00000	0.96488	0.43271	0.90539
	9. *CNSTA*	0.22391	1.00000	1.00000	0.47985	0.26842	0.68382
	10. *LBSTA*	0.79350	0.58819	1.00000	0.30777	0.61686	0.92698
	11. *SBSTA*	0.42189	0.04336	1.00000	0.11786	0.21060	0.34509
	12. *SPSTA*	0.00520*	0.04834	0.00470*	0.78930	0.62082	0.48170
KarnChanaburi	13. *KBSTA*	1.00000	0.54486	1.00000	0.41940	0.96118	0.01267
	14. *PBSTA*	0.96993	1.00000	1.00000	0.23212	0.05442	0.77832

Table 4.3 (cont.)

Group	Population	*Pgon17-69*	*Pgon17-75*	*Pgon17-79*	*Pgon69-75*	*Pgon69-79*	*Pgon75-79*
North-east region							
Khonkaen	15. *KKSTA*	0.47173	0.62738	0.07102	0.92229	0.88278	0.82295
	16. *BRSTA*	1.00000	0.47172	0.11248	0.78841	0.24545	0.30289
	17. *KSSTA*	0.04832	0.75145	0.62627	0.56986	0.21611	0.68333
	18. *MSSTA*	0.71013	0.57715	0.15170	0.36885	0.24875	0.62201
	19. *NRSTA*	0.07280	0.30609	1.00000	0.26919	0.96916	0.73906
Udornthani	20. *UDSTA*	0.34225	0.82576	1.00000	0.43236	0.13356	0.47328
	21. *NKSTA*	0.25907	0.14289	0.08737	0.90658	0*	0.53401
	22. *NPSTA*	0.83134	0.86603	0.96558	0.04345	0.16710	0.03332
	23. *SNSTA*	0.29542	0.02133	0*	0.72868	0.51154	0.24558
Ubolratchathani	24. *UBSTA*	0.03313	0.14552	0.60717	0.12590	0.81632	0.34212
	25. *MDSTA*	0.35898	0.34504	0.45434	0.89661	0.62671	0.98055
	26. *RESTA*	0.98044	0.23886	0.85639	0.22805	0.50638	0.66984
	27. *SKSTA*	0.39125	0.06411	0.87572	0.51905	0.17564	0.74536
	28. *SRSTA*	0.07318	0.09862	0.34098	0.60197	0.38823	0.62569
	29. *YSSTA*	0.43800	0.25159	0.85540	0.47003	0.61201	0.16635

Table 4.4 Genetic variability at 4 loci in all fisheries stations stocks. Significant difference between the direct-count and expected heterozygosities is tested using the independent sample *t*-test (Archie, 1985).

Population	Mean no. of alleles per locus	Effective number of alleles	Mean heterozygosity		F_{IS}
			Direct-count	HdyWbg expected	
Central group					
1.PLSTA	12.8±3.8	7.082	0.743±0.060	0.827±0.042	0.1016
1.TKSTA	10.5±3.0	4.829	0.783±0.042	0.779±0.035	-0.0051
1.UTSTA	11.8±2.4	6.311	0.805±0.077	0.816±0.043	0.0135
2.NSSTA	10.5±2.9	5.962	0.785±0.038	0.803±0.041	0.0224
2.PCSTA	12.3±4.4	8.152	0.801±0.063	0.830±0.053	0.0349
2.KPSTA	12.3±2.7	6.578	0.852±0.046	0.829±0.035	-0.0277
3.AYSTA	11.3±3.7	6.669	0.801±0.057	0.815±0.046	0.0172
3.ATSTA	12.3±4.1	6.964	0.795±0.066	0.810±0.052	0.0185
3.CNSTA	12.3±3.4	6.703	0.750±0.058	0.803±0.056	0.0660
3.LBSTA	11.3±3.7	6.559	0.760±0.090	0.784±0.064	0.0306
3.SBSTA	12.0±3.5	5.648	0.800±0.078	0.800±0.047	0
3.SPSTA	10.8±3.0	6.605	0.820±0.050	0.807±0.052	-0.0161
4.KBSTA	12.0±3.2	6.176	0.820±0.049	0.800±0.053	-0.0250
4.PBSTA	11.3±3.8	5.711	0.770±0.083	0.775±0.068	0.0065
North-east group					
5.KKSTA	10.0±1.7	3.501	0.779±0.044	0.765±0.040	-0.0183
5.BRSTA	10.8±2.6	6.172	0.821±0.039	0.812±0.042	-0.0111
5.KSSTA	9.0±2.0	3.501	0.688±0.015	0.716±0.027	0.0391
5.MSSTA	9.3±2.4	4.145	0.781±0.024	0.762±0.020	-0.0249
5.NRSTA	11.5±4.5	7.063	0.845±0.040	0.811±0.049	-0.0419
6.UDSTA	10.8±2.8	5.815	0.790±0.021	0.817±0.032	0.0330
6.NKSTA	10.5±2.3	5.245	0.794±0.029	0.798±0.032	0.0050
6.NPSTA	8.8±2.2	3.302	0.686±0.032	0.694±0.033	0.0115
6.SNSTA	10.5±2.7	4.160	0.729±0.033	0.766±0.010	0.0483
7.UBSTA	10.3±2.3	5.817	0.830±0.047	0.814±0.033	-0.0197
7.MDSTA	10.3±2.6	4.289	0.734±0.055	0.754±0.049	0.0265
7.RESTA	11.0±2.9	4.286	0.749±0.012	0.773±0.010*	0.0310
7.SKSTA	10.3±2.7	5.188	0.806±0.031	0.796±0.036	-0.0126
7.SRSTA	11.5±3.4	4.560	0.765±0.043	0.785±0.016	0.0255
7.YSSTA	10.0±3.0	4.545	0.768±0.030	0.782±0.020	0.0179

* $P < 0.05$

Table 4.5 Contingency test of differentiation among *Puntius gonionotus* stock within DOF central group (according to Table 4.1). Chi-square was a Fisher's combination of *P*-values method for all loci (Sokal & Rohlf, 1981). Significant levels were adjusted for multiple tests within group using a sequential Bonferroni technique (Hochberg, 1988; Rice, 1989).

Populations	*Pgon-17*	*Pgon-69*	*Pgon-75*	*Pgon-79*	χ^2
1. *Phitsanulok*					
PLSTA-TKSTA	0.00272*	0.41142	0.79052	0*	****
PLSTA-UTSTA	0.02092	0.27795	0.00647*	0.03873	26.878*
TKSTA-UTSTA	0*	0.07287	0.00350*	0*	****
2. *Nakornsawan*					
NSSTA-PCSTA	0.01306	0.01626	0.00318*	0.00149*	41.434*
NSSTA-KPSTA	0.00802	0.19334	0.09169	0*	****
KPSTA-PCSTA	0.05291	0.08185	0.02785	0.00184*	30.641*
3. *Ayutthaya*					
AYSTA-ATSTA	0.01321	0.94149	0.19227	0.09142	16.856
AYSTA-CNSTA	0.00221	0.00867	0.00223	0*	****
AYSTA-LBSTA	0.00921	0.00235	0.00093*	0.00043*	50.945*
AYSTA-SBSTA	0.01099	0.01290	0.00962	0*	****
AYSTA-SPSTA	0*	0.29389	0.00085*	0*	****
ATSTA-CNSTA	0*	0.10668	0.36680	0.00003*	****
ATSTA-LBSTA	0.21099	0.00126	0.02719	0.02439	31.102*
ATSTA-SBSTA	0.03280	0.09013	0.08530	0.00210	28.902*
ATSTA-SPSTA	0.13868	0.52275	0.30147	0.00564	18.002
CNSTA-LBSTA	0.18175	0.11314	0.04594	0.24178	16.768
CNSTA-SBSTA	0.00240	0.70115	0.00597	0.03890	29.510*
CNSTA-SPSTA	0.00113	0.03216	0.85423	0.01744	28.858*
LBSTA-SBSTA	0.04255	0.12356	0.28262	0.17674	16.489
LBSTA-SPSTA	0.41231	0.00117	0.03414	0.05642	27.777*
SBSTA-SPSTA	0.00226	0.21726	0.00315	0.02748	33.947*
4. *Karnchanaburi*					
KBSTA-PBSTA	0.39887	0.48991	0.81202	0.29484	6.1244

* significant at $\alpha = 0.05$ after a sequential Bonferroni correction
**** *P*-value > 0.05

Table 4.5 (cont.) Contingency test of differentiation among stations within DOF north-east group.

Populations	*Pgon-17*	*Pgon-69*	*Pgon-75*	*Pgon-79*	χ^2
5. *Khonkaen*					
KKSTA-BRSTA	0*	0*	0.00754	0.08491	****
KKSTA-KSSTA	0*	0.42150	0.00121*	0.19517	****
KKSTA-MSSTA	0*	0.19803	0.00004*	0.39183	****
KKSTA-NRSTA	0*	0*	0.01568	0*	****
BRSTA-KSSTA	0*	0*	0*	0.00967	****
BRSTA-MSSTA	0*	0.00094*	0*	0.56780	****
BRSTA-NRSTA	0.00518*	0.25228	0.06003	0.01179	27.787*
GSSTA-MSSTA	0.01273	0.06026	0.00041*	0.15825	33.631*
GSSTA-NRSTA	0*	0*	0*	0*	****
MSSTA-NRSTA	0*	0*	0.00027*	0.00062*	****
6. *Udornthani*					
UDSTA-NKSTA	0*	0.00889	0.65371	0*	****
UDSTA-SNSTA	0.00133*	0.00009*	0.00074*	0*	****
UDSTA-NPSTA	0*	0*	0*	0*	****
NKSTA-SNSTA	0*	0.00464*	0.03800	0.00021*	****
NKSTA-NPSTA	0*	0.00032*	0.00367*	0*	****
SNSTA-NPSTA	0.45664	0.25663	0.59284	0.00057*	20.273*
7. *Ubolratchathani*					
UBSTA-RESTA	0*	0*	0.01998	0*	****
UBSTA-MDSTA	0*	0.03101	0.00107*	0*	****
UBSTA-SKSTA	0*	0*	0.00244	0*	****
UBSTA-SRSTA	0*	0*	0.01048	0*	****
UBSTA-YSSTA	0*	0.00200	0.04378	0*	****
RESTA-MDSTA	0.17727	0.04677	0.11973	0.06604	19.265*
RESTA-SKSTA	0*	0.30722	0.02556	0.00799	****
RESTA-SRSTA	0*	0.31227	0.01448	0.00913	****
RESTA-YSSTA	0.86846	0.33541	0.97112	0.98724	2.5511
MDSTA-SKSTA	0*	0.00005*	0.08181	0.03112	****
MDSTA-SRSTA	0*	0.36984	0.13648	0*	****
MDSTA-YSSTA	0.30936	0.02985	0.09621	0.04589	20.215*
SKSTA-SRSTA	0*	0.01770	0.00363	0.04605	****
SKSTA-YSSTA	0*	0.00932	0.00203	0.01991	****
SRSTA-YSSTA	0*	0.04286	0.04519	0.00483	****

* significant at $\alpha = 0.05$ after a sequential Bonferroni correction

**** *P*-value > 0.05

Table 4.6 Contingency test between contiguous pair (see Fig. 4.3). Chi-square was a Fisher's combination of *P*-values metod for all loci (Sokal & Rohlf, 1981). Significant levels were adjusted for multiple tests within group using a sequential Bonferroni technique (Hochberg, 1988; Rice, 1989).

Populations	*Pgon-17*	*Pgon-69*	*Pgon-75*	*Pgon-79*	χ^2
Central group					
PLSTA-UTSTA	0.02092	0.27795	0.00647	0.03873	26.878*
PLSTA-KPSTA	0.21918	0.96802	0.22068	0.00021*	23.060*
PLSTA-PCSTA	0.34783	0.04181	0.07732	0.00446*	24.406*
KPSTA-TKSTA	0*	0.10343	0.23352	0*	****
NSSTA-PCSTA	0.01306	0.01626	0.00318*	0.00149*	41.434*
NSSTA-CNSTA	0.04463	0.05745	0.02948	0.00057*	33.920*
CNSTA-SBSTA	0.00240*	0.70115	0.00597*	0.03890	29.510*
SBSTA-LBSTA	0.04255	0.12356	0.28262	0.17674	16.489
ATSTA-SBSTA	0.03280	0.09013	0.08530	0.00210*	28.902*
ATSTA-SPSTA	0.13868	0.52275	0.30147	0.00564	18.002
AYSTA-ATSTA	0.01321	0.94149	0.19227	0.09142	16.856
KBSTA-SPSTA	0.00292*	0.21699	0.12415	0.05998	24.528*
KBSTA-PBSTA	0.39887	0.48991	0.81202	0.29484	6.1244
North-east group					
UDSTA-NKSTA	0*	0.00889*	0.65371	0*	****
KKSTA-UDSTA	0*	0.01395*	0.00002*	0*	****
KKSTA-MSSTA	0*	0.19803	0.00004*	0.39183	****
KSSTA-MSSTA	0.01273	0.06026	0.00041*	0.15825	33.631*
RESTA-MSSTA	0.40492	0.26368	0.05274	0.48208	11.818
SNSTA-NPSTA	0.45664	0.25663	0.59284	0.00057*	20.273*
KSSTA-SNSTA	0.17443	0.26254	0.00286*	0.00093*	31.841*
MDSTA-SNSTA	0.00882	0.02212	0.16417	0.03243	27.555*
MDSTA-YSSTA	0.30936	0.02985	0.09621	0.04589	20.215*
RESTA-YSSTA	0.86846	0.33541	0.97112	0.98724	2.5511
SKSTA-YSSTA	0*	0.00932	0.00203*	0.01991	****
SKSTA-SRSTA	0*	0.01770	0.00363*	0.04605	****
UBSTA-SKSTA	0*	0*	0.00244*	0*	****
BRSTA-NRSTA	0.00518*	0.25228	0.06003	0.01179*	27.787*
BRSTA-SRSTA	0*	0.00005*	0.00006*	0.06626	****

* significant at $\alpha = 0.05$
**** *P*-value > 0.05

Table 4.7 Contingency test of differentiation among transferred stocks. Years of transferring were shown in parentheses Chi-square was a Fisher's combination of *P*-values metod for all loci (Sokal & Rohlf, 1981). Significant levels were adjusted for multiple tests using a sequential Bonferroni technique (Hochberg, 1988; Rice, 1989).

Populations	*Pgon-17*	*Pgon-69*	*Pgon-75*	*Pgon-79*	χ^2
NKSTA-KKSTA (72)	0*	0.08639	0.00309	0*	****
MSSTA-KSSTA (81)	0.01273	0.06026	0.00041*	0.15825	33.631*
PCSTA-TKSTA (81)	0*	0.13638	0.01599	0.00130	****
KSSTA-BRSTA (85)	0*	0*	0*	0.00967	****
KBSTA-PBSTA (86)	0.39887	0.48991	0.81202	0.29484	6.1244
RESTA-MDSTA (87)	0.17727	0.04677	0.11973	0.06604	19.265
UBSTA-MDSTA (87)	0*	0.03101	0.00107	0*	****
YSSTA-MDSTA (87)	0.30936	0.02985	0.09621	0.04589	20.215*
KSSTA-YSSTA (88)	0.27995	0.07743	0.01843	0.02605	22.946*
PCSTA-UTSTA (89)	0.00406	0.01875	0.14556	0.01146	31.758*
TKSTA-UTSTA (89)	0*	0.07287	0.00350	0*	****
KSSTA-UTSTA (89)	0*	0.03992	0*	0.00007*	****
PCSTA-CNSTA (90)	0.01034	0.03703	0.03867	0.06356	27.752*
MSSTA-LBSTA (90)	0*	0*	0*	0*	****
MSSTA-SBSTA (90)	0*	0*	0.00333	0*	****
SRSTA-SKSTA (90)	0*	0.01770	0.00363	0.04605	****
SPSTA-ATSTA (91)	0.13868	0.52275	0.30147	0.00564	18.002
UTSTA-KPSTA (91)	0.50135	0.23524	0.00632	0.11746	18.686
SPSTA-LBSTA (91)	0.41231	0.00117	0.03414	0.05642	27.777*
KSSTA-NPSTA (91)	0.00600	0.00870	0.00170	0.00972	41.742*
NKSTA-SRSTA (91)	0.00079	0.00716	0.02036	0.00509	42.514*
AYSTA-ATSTA (92)	0.01321	0.94149	0.19227	0.09142	16.856
UDSTA-NPSTA (92)	0*	0*	0*	0*	****
KSSTA-NSSTA (93)	0*	0.00417	0*	0.00032*	****
ATSTA-SBSTA (94)	0.03280	0.09013	0.08530	0.00210	28.902*

* significant at $\alpha = 0.05$ after a sequential Bonferroni correction

**** *P*-value > 0.05

Table 4.8 F-statistics (± SE) among *Puntius gonionotus* hatchery stocks in the central and north-east groups. Bootstrapping procedure was used to construceted 95% confidence interval for estimates over all loci.

Locus	$F(F_{IT})$	$f(F_{IS})$	θ (F_{ST})
All stations			
Pgon-17	0.0637±0.0143*	0.0158±0.0106	0.0486±0.0687
Pgon-69	0.0428±0.0160*	0.0141±0.0162	0.0291±0.0245
Pgon-75	0.0377±0.0132*	0.0175±0.0132	0.0205±0.0122
Pgon-79	0.0415±0.0105*	-0.0001±0.0093	0.0416±0.0504
Mean	0.0475±0.0065*	0.0119±0.0040*	0.0360±0.0065*
95% CI:	0.0389 to 0.0593	0.0042 to 0.0167	0.0247 to 0.0454
Central group			
Pgon-17	0.0157±0.0161	0.0011±0.1680	0.0146±0.0030*
Pgon-69	0.0461±0.0288	0.0405±0.0290	0.0058±0.0005*
Pgon-75	0.0505±0.0179*	0.0366±0.0179*	0.0144±0.0029*
Pgon-79	0.0154±0.0111	-0.0027±0.0128	0.0181±0.0046*
Mean	0.0299±0.0095*	0.0165±0.0112	0.0136±0.0024*
95% CI:	0.0156 *to* 0.0484	-0.0007 *to* 0.0385	0.0085 *to* 0.0172
North-east group			
Pgon-17	0.0916±0.0179*	0.0312±0.0119*	0.0623±0.0582
Pgon-69	0.0182±0.0139	-0.0088±0.0155	0.0268±0.0108*
Pgon-75	0.0171±0.0173	-0.0001±0.0186	0.0173±0.0045*
Pgon-79	0.0424±0.0164*	0.0027±0.0140	0.0398±0.0238
Mean	0.0447±0.0188*	0.0071±0.0093	0.0378±0.0104*
95% CI:	0.0177 *to* 0.0751	-0.0060 to 0.0240	0.0220 to 0.0542

* significant at α =0.05 as approximated 95% CI (estimate ± 2SE)

Table 4.9 Pairwise Cavalli-Sforza and Edwards (1967) chord distances (below diagonal) and geographic distances in km. (above diagonal) between populations of *Puntius gonionotus* from 29 fisheries stations. Refer to Table 4.1 for population identifications.

POP	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
1	--	138	118	129	73	103	301	270	193	259	230	477	505	500	319	608	396	392	457	434	485	617	524	601	562	432	666	655	503
2	.270	--	179	185	157	68	357	326	249	315	286	526	554	549	457	664	533	530	513	572	623	755	662	739	700	570	804	711	641
3	.255	.312	--	247	189	177	419	388	311	377	348	591	619	614	437	726	514	510	575	552	603	735	642	719	680	550	784	773	621
4	.280	.304	.270	--	113	117	172	141	64	130	101	340	368	363	493	478	525	521	327	563	614	746	653	697	710	562	639	525	599
5	.239	.310	.272	.294	--	90	285	254	177	243	214	444	472	467	352	592	429	423	441	467	518	650	557	634	595	463	699	639	536
6	.235	.319	.222	.300	.267	--	289	258	181	247	218	458	486	481	422	596	499	495	445	537	588	720	627	704	849	535	757	643	606
7	.286	.299	.334	.353	.308	.343	--	31	121	98	71	176	204	199	405	365	475	431	215	520	571	696	603	582	598	450	527	413	484
8	.227	.267	.286	.316	.274	.313	.230	--	92	67	40	205	233	228	400	361	470	426	210	515	566	691	598	580	593	465	522	408	482
9	.287	.308	.266	.270	.254	.307	.318	.294	--	85	53	294	322	317	448	431	540	496	280	585	636	751	668	650	653	515	592	478	552
10	.249	.298	.268	.260	.261	.293	.301	.260	.219	--	33	253	281	276	388	349	458	414	198	503	554	679	586	568	679	433	510	396	470
11	.222	.257	.292	.307	.269	.295	.286	.250	.263	.232	--	242	270	265	421	382	491	447	231	536	587	712	619	601	614	466	543	429	503
12	.253	.278	.281	.270	.245	.287	.319	.239	.247	.230	.260	--	91	204	549	510	619	575	359	664	715	840	747	729	742	612	674	557	631
13	.256	.247	.269	.286	.296	.308	.273	.236	.243	.226	.237	.249	--	144	577	538	647	603	387	692	743	868	775	757	770	640	702	585	659
14	.252	.254	.293	.315	.292	.313	.279	.253	.269	.230	.215	.255	.198	--	572	533	642	598	382	687	738	863	770	752	765	635	697	580	654
15	.345	.388	.332	.363	.411	.348	.437	.412	.419	.406	.363	.396	.385	.402	--	200	77	73	190	115	166	298	205	282	243	113	347	256	184
16	.273	.321	.274	.273	.295	.287	.302	.280	.287	.270	.276	.288	.272	.275	.376	--	184	145	151	315	366	414	321	289	302	146	225	111	191
17	.345	.371	.324	.320	.410	.302	.426	.413	.375	.359	.370	.390	.368	.399	.325	.330	--	44	260	192	243	221	128	216	166	47	279	184	118
18	.329	.363	.321	.302	.395	.332	.390	.383	.375	.327	.330	.359	.351	.359	.316	.306	.247	--	216	188	239	265	172	213	210	40	274	177	115
19	.216	.254	.253	.289	.230	.271	.260	.207	.248	.218	.205	.227	.206	.207	.370	.267	.361	.317	--	305	356	481	388	370	383	235	312	198	272
20	.307	.352	.268	.273	.347	.286	.384	.346	.318	.293	.346	.336	.334	.367	.398	.277	.305	.317	.311	--	51	252	159	401	278	228	462	373	303
21	.316	.377	.349	.376	.407	.374	.388	.352	.408	.395	.367	.406	.345	.387	.306	.379	.380	.358	.350	.378	--	303	210	448	329	279	513	422	350
22	.380	.416	.353	.363	.477	.388	.448	.420	.434	.408	.411	.421	.398	.426	.350	.390	.261	.288	.408	.366	.383	--	93	271	104	268	332	350	215
23	.321	.383	.295	.312	.415	.322	.385	.365	.371	.327	.348	.361	.346	.369	.322	.303	.238	.228	.343	.287	.348	.22	--	286	119	175	347	312	230
24	.295	.323	.271	.314	.315	.313	.371	.339	.333	.317	.299	.312	.315	.346	.326	.339	.376	.355	.287	.311	.368	.366	.326	--	167	169	61	227	98
25	.312	.354	.298	.291	.390	.276	.407	.376	.379	.347	.347	.362	.359	.376	.299	.317	.213	.222	.326	.312	.367	.260	.244	.349	--	162	228	246	111
26	.285	.351	.285	.291	.369	.280	.367	.361	.358	.321	.325	.353	.325	.330	.329	.267	.242	.194	.299	.271	.349	.317	.232	.352	.227	--	230	137	71
27	.363	.405	.335	.375	.393	.352	.395	.399	.398	.380	.380	.403	.350	.398	.287	.336	.351	.315	.343	.364	.315	.377	.311	.354	.334	.312	--	143	159
28	.328	.406	.346	.382	.394	.344	.430	.372	.427	.368	.363	.414	.375	.410	.296	.373	.367	.344	.353	.349	.281	.388	.328	.346	.346	.336	.287	--	135
29	.282	.323	.265	.258	.346	.294	.357	.344	.335	.290	.320	.337	.307	.306	.325	.250	.241	.221	.291	.283	.340	.312	.231	.337	.225	.159	.323	.344	--

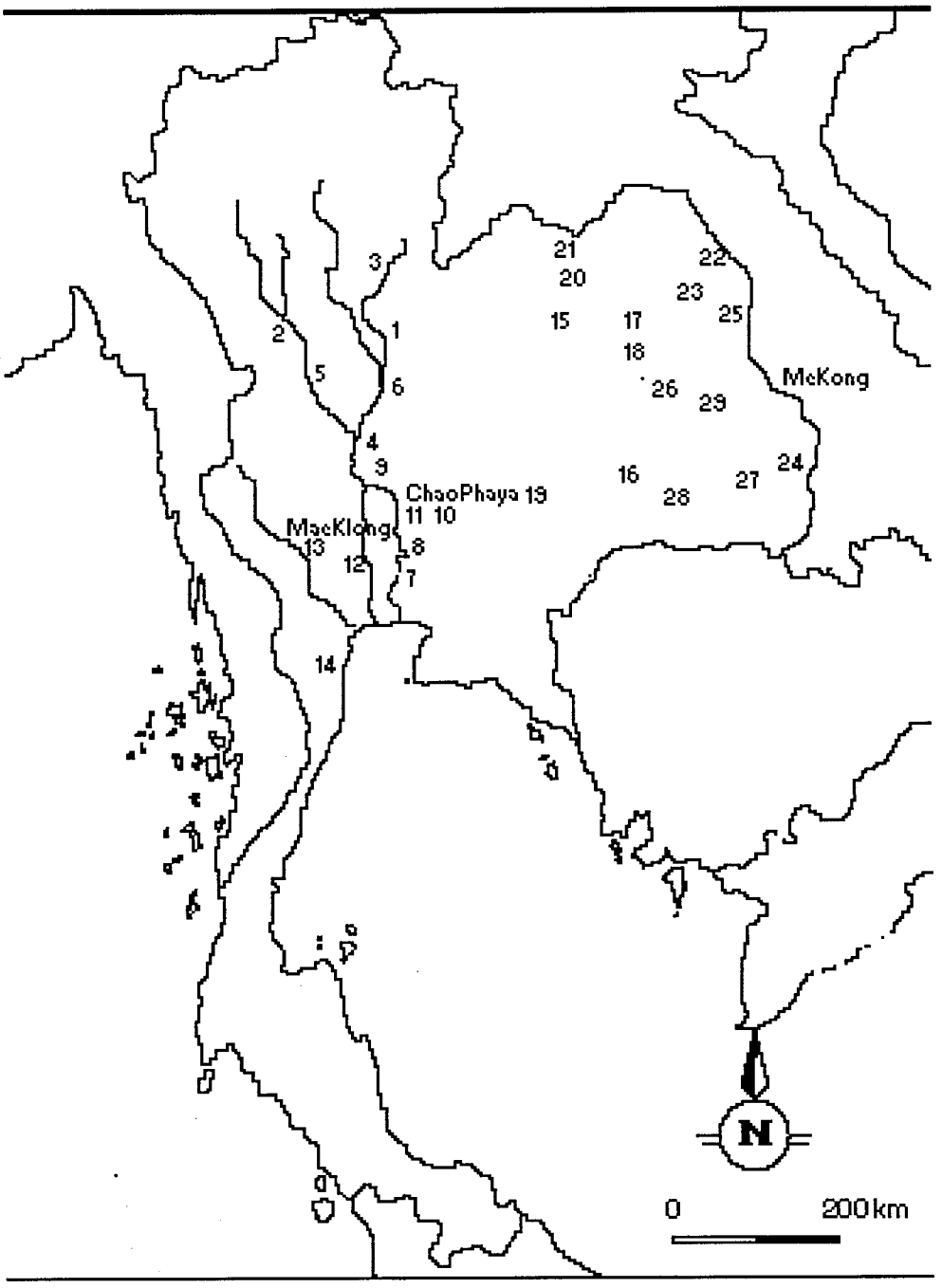


Figure 4.1 Sample sites of fisheries stations where the *Puntius gonionotus* stocks were studied in this study. Numbers refer to location in Table 4.1.

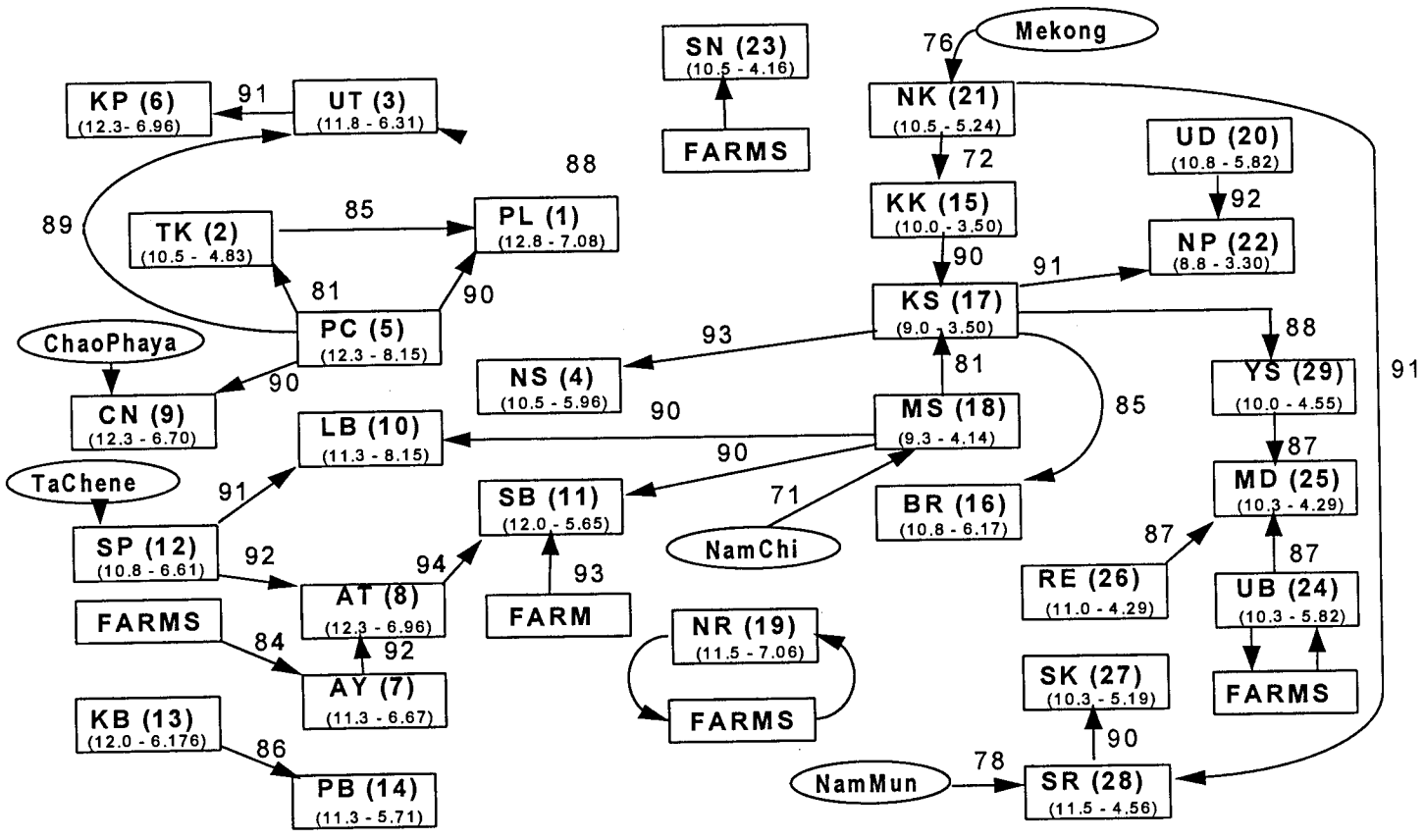


Figure 4.2 *Puntius gonionotus* stock transfer (partial) history. Diagram was reconstructed using information obtained from interview with station personnel. Arrows indicate directions of transferring, and numbers are the year of transfer. Two numbers in parenthesis indicated the average number of alleles per locus and effective number of alleles.

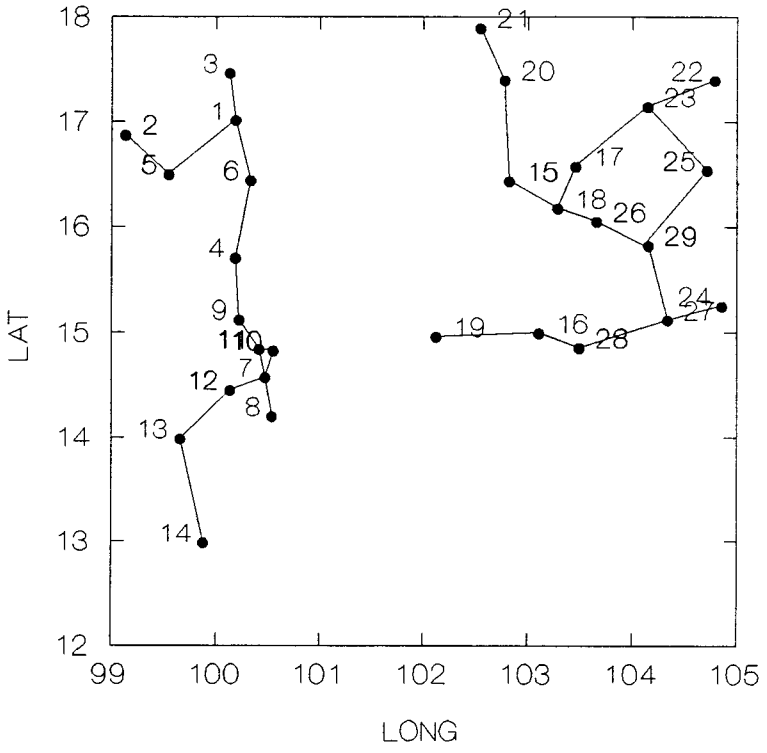


Figure 4.3 Garbriel network (Garbriel & Sokal, 1969) of the Fisheries Stations in Thailand. The station numbers refer to Table 4.1. Contiguous localities are indicated by lines connecting the stations numbers.

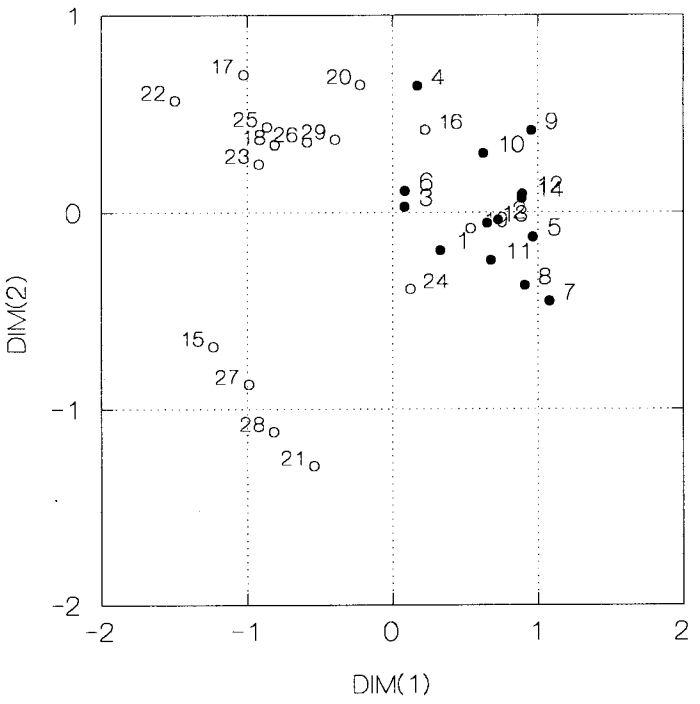


Figure 4.4 Genetic relationships among the studied population of *Puntius gonionotus* in Fisheries Stations in Thailand. The plot is against dimension 1 and 2 of the configuration produced by a multidimensional scaling (MDS) analysis of Cavalli-Sforza and Edward chord distances. Closed circles represent Northeast region, open circles represent the Central region. Numbers refer to stations in Table 4.1.

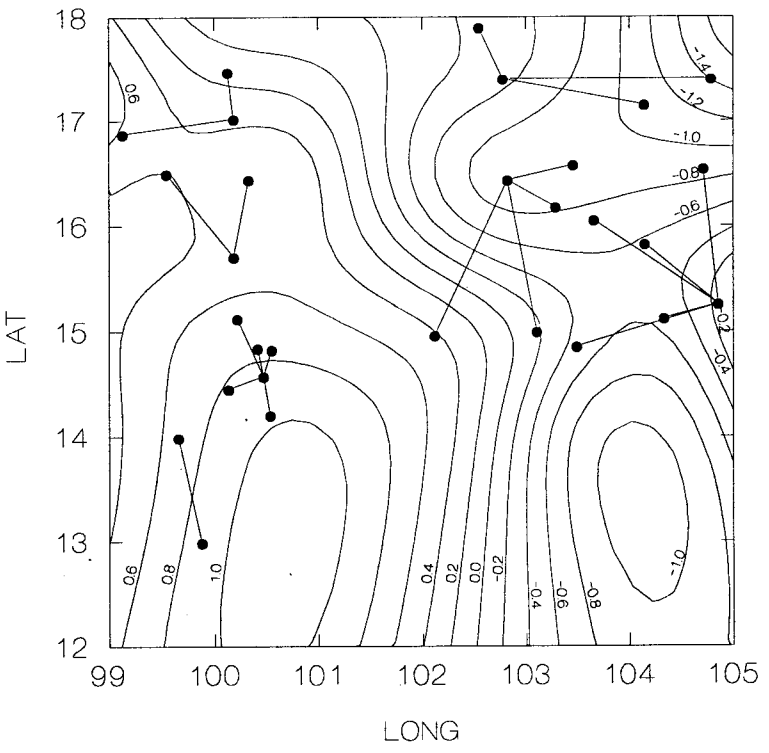


Figure 4.5 Contour plot of the first dimension of the nonmetric multidimensionsal scaling of pairwise Cavalli-Sforza and Edwards (1967) chord distances among *Puntius gonionotus* populations in Fisheries Stations in Thailand. Also shown, the network of station grouping according to the DOF administration (Table 4.1).

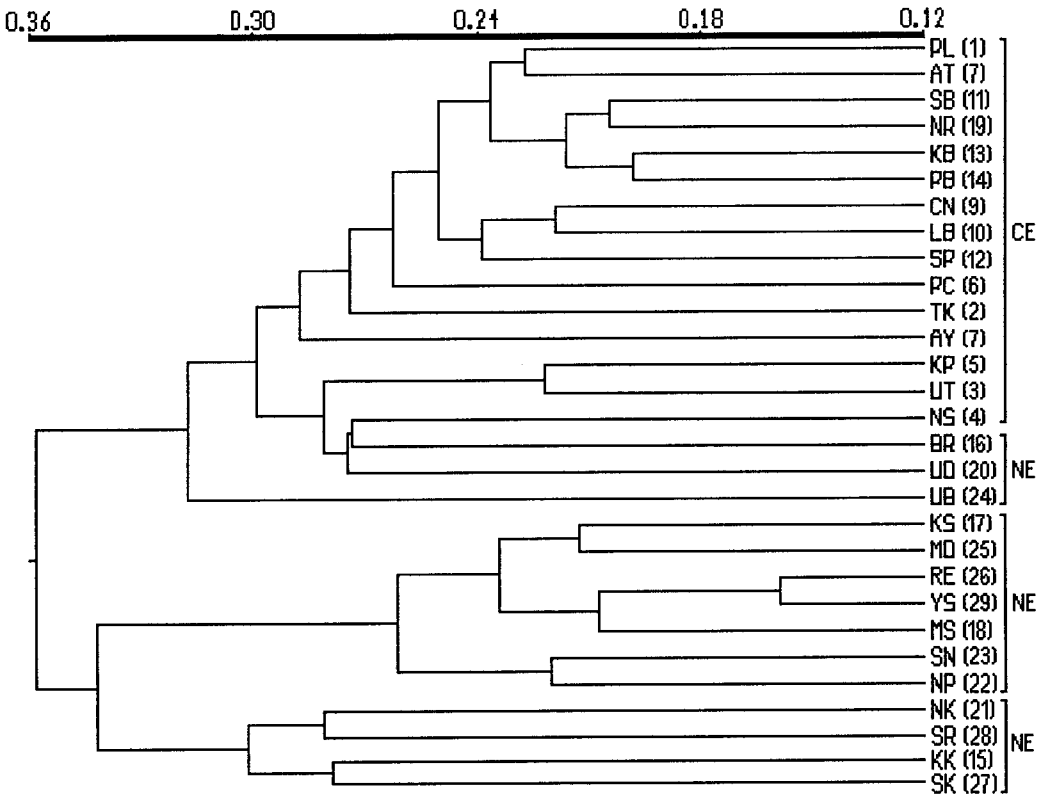


Figure 4.6. UPGMA dendogram clustering Cavalli-Sforza and Edwards (1967) chord distances among hatchery stocks of *Puntius gonionotus* in Thai Fisheries Stations. CE is the central group and NE is the northeast group. Numbers refer to sites in Table 4.1.

Chapter 5

Use of microsatellite DNA markers in genetic management of the hatchery populations of ***Puntius gonionotus*** (Bleeker)

ABSTRACT

The potential of parental identification during large-scale communal rearing was assessed using microsatellite DNA markers. The study was conducted in three hatchery populations of *Puntius gonionotus* (Bleeker) in Thailand. A two-level nested mating design was used in which four sires were mated to each of four dams to produce four half-sib and 16 full-sib families in each population. All families of each population were grown together from birth and samples of the largest as well as of randomly chosen individuals were taken after a 3-month growth period. The parentage of both types of samples was successfully established (99.89%) using one to five microsatellite makers generated from the same species. There were significant differences among sires and dams for the growth and survival of their progeny. Heritability estimates from maternal half-sib for the three populations ranged from 0.290 to 0.523 and 0.193 to 0.421 for standard length and body weight, respectively. A strong negative relationship was observed between the effective number of alleles per locus and additive genetic variance, and between the effective number of alleles per locus and total phenotypic variance. This was simply coincidence as only three populations were studied. No relationships were observed among additive genetic variance, phenotypic variance and the observed heterozygosity of the populations. The results of this study suggest applications of microsatellite markers in genetic management of hatchery populations including controlling inbreeding, maintaining genetic variability, and selective breeding.

INTRODUCTION

Large numbers of fish are being produced from hatcheries throughout the world for release into natural water to supplement natural production and for aquaculture purposes. In Thailand, over 50 million *Puntius gonionotus* (Bleeker) were produced from government hatcheries alone, and more than half of this number were used for restocking. There has been an increasing doubt of the benefits of such a program as it may pose potential problems for wild populations (Hindar *et al.*, 1991; Krueger & May, 1991; Ryman, 1991; Waples, 1991). Some of the concerns include changes in allele frequencies and loss of genetic variation through genetic drift, and adaptation to culture conditions through selection (Allendorf, 1993). Therefore, genetic management for restocking and rehabilitation programs should aim to maintain genetic uniqueness and genetic variation of the population(s) after founding.

On the other end of hatchery production, the breeding program for aquaculture aims to "...*change the average performance of a population in a defined direction to the benefit of industry and the consumer market*" (Gjedrem, 1993). Selective breeding in fishes is still at its early stage relative to the domesticated animals. Only a few genetically improved strains of fishes have been developed for commercial use (Main & Reynolds, 1993), despite its greater potential of genetic improvement as has been seen in domesticated farm animals (Wilkins, 1981). Several selective breeding methods used in domesticate terrestrial farm animals have been employed in fishes with varying results. The major contributing problem, perhaps, is the fact that the genetic features and natural behavior of fishes are still relatively unknown. Unfortunately such studies are limited due to the fact that physical tagging of fish at the fry stage is impossible.

Researchers are forced to conduct their experiments in separate ponds or cages, where full sib families are grown together for the length of the experiment or until they can be tagged. Besides being space and labor intensive, the results are largely confounded by both common environment (within ponds or cages) and replicate variances of unpredictable magnitudes and unknown distributions (Uraiwan & Doyle, 1986).

Microsatellite DNA markers possess several characteristics, such as a large number of loci and high variability which make them a potentially important tool for individual identification and kinship studies (Queller & Strassmann, 1993; Wright, 1993). Recently, microsatellites has been successfully used to determine the pedigree of a mixed rainbow trout population (Herbinger *et al.*, 1995). Ability to identify individuals allows all family groups to be reared together from birth so the experimental design is simplified and unwanted variances are eliminated. Knowledge of kinship and pedigree information allows geneticists to carry out a proper management and employ sophisticated selection approaches in the populations.

In this paper, the potential of parental identification was examined in the large communal rearing of *P. gonionotus*. By using pedigree information, several genetic aspects of the species were assessed, including quantitative genetic variation, survivability and growth of the populations.

MATERIALS AND METHODS

Studied populations and breeding procedure

Three hatchery populations of *P. gonionotus* from the northern region of Thailand, including Phitsanulok Fisheries Development Center, Phichit and Tak Fisheries Stations were studied. The rearing experiment took place at the Phitsanulok Fisheries Development Center of the Department of Fisheries, Phitsanulok province, Thailand. Ripe females and males were chosen at random from each station broodstock and transported from Phichit and Tak Fisheries Stations to the Phitsanulok Center a few days before artificial breeding. The mating design was a nested cross in which four males were mated to one female and a total of four females (4 maternal half-sib and 16 full-sib families) were used for each population. Blood samples were collected from the parents after spawning, and were used for parental references in assigning the parentage of communal rearing offspring.

In the breeding procedure, for each stock four ripe females were induced to spawn by hormone (homogenized common carp pituitary gland) injection. Thereafter, eggs were removed by abdominal compression, divided into four batches (approximately 20 ml each batch), and placed in separate dry containers. Each batch was fertilized with sperm removed from a male chosen at random from the same stock as the female. After water absorption, fertilized eggs were pooled and hatched in a water circulated tank. The hatching period was about 8 -12 hours. Approximately two days after the hatch, the fry were transferred to an outdoor 70 sq.m. concrete pond for further grow-out. The fish in each pond were randomly thinned to approximately 5,000 fish after 30 day of age. These fish were grown for another 60 days.

At the end of the experiment, two groups of 300 fish, *i.e.*, random chosen fish and the largest fish (upper tail of the distribution), were chosen from each stock. The sampled fish were killed by subjection to an over-dose of MS-222. Standard length and body weight were measured on each individual. Tissue samples were collected from the anterior area below the dorsal fin and preserved in individual vials containing 100% ethanol and were used for family identification by microsatellite DNA analysis.

Genotyping and parental identification

DNA was extracted from the samples and subjected to PCR amplification using the microsatellite primers that were developed from the *P. gonionotus* genomic library. DNA extraction and PCR procedures have been previously described (*see* Chapter 2). Genotypes of individuals were scored relative to a sequencing ladder generated from single-stranded M-13 DNA (Yanisch-Perron *et al.*, 1985).

One to five single-locus microsatellite DNA markers (*Pgon-17*, *Pgon-31*, *Pgon-69*, *Pgon-75* and *Pgon-79*) were used to genotype all parents and offspring. Genotype profiles of offspring were compared to their potential parents to match the relationships. In the case of unexpected or novel genotypes, the samples were re-amplified to verify such events.

Statistical analyses

(a) *Progeny growth and survival rate:*

Size-at-3 months (length and weight) of progeny from the various dams and sires were compared by analysis of variance. The statistical model used in the test was a two-level nested anova (Sokal & Rohlf, 1981):

$$Y_{ijk} = \mu + D_i + S_{j(i)} + e_{ijk}$$

where

Y_{ijk} is the *k*th observation in the *j*th sire of the *i*th dam,

μ is the overall mean,

D_i is the random contribution for the *i*th dam,

$S_{j(i)}$ is the random contribution for the *j*th sire of the *i*th dam,

e_{ijk} is the residual effect.

Length and weight were normalized using the Box-Cox transformation procedure (Sokal & Rohlf, 1981).

The number of progeny from the various dams was compared by a one-way anova. Number of progeny were normalized with a square root transformation in all tests, except for the random group of Phitsanulok stock in which reciprocal transformation ($1/N_{OFF}$) was used.

Post-hoc test was conducted to test significant differences of means (length, weight, and number of progeny) between dams in each station stock. A Bonferroni correction of *P*-value was applied for overall multiple test.

All tests were carried out using the SYSTAT computer program (Wilkinson *et al.*, 1992).

(b) *Effective number of population:*

The effective number of breeders in the populations were estimated in two ways, *i.e.*, (1) the variance effective population number (Crow & Denniston, 1988) and (2) the inbreeding effective population size (Lande & Barrowclough, 1987). Here the estimates

followed the formulae described in Hedrick *et al.* (1995). Inbreeding effective number is defined as the reciprocal of the probability that two randomly chosen gametes come from the same parents while the variance effective number is defined as the amount of allele frequency drift per generation as measured by its variance (Crow & Kimura, 1970).

The variance effective population size was estimated as (Crow & Denniston, 1988)

$$N_{ec(v)} = \frac{4N_f N_m}{xN_f + yN_m},$$

where

$$x = f + m\sigma^2_{km}/\bar{k}_m \text{ and } y = m + f\,\sigma^2_{kf}/\bar{k}_f,$$

where N_f and N_m are the actual numbers of breeding females and males, $\bar{k}_f$ and σ^2_{kf} are the mean and variance of the number of progeny produced by females, $\bar{k}_m$ and σ^2_{km} are the mean and variance of the number of progeny produced by males, *f* and *m* are the proportions of females and males progeny at the same age (spawning age), where *f* + *m* = *1*. Here the objective was to compare the effective size between groups that chosen at random and the large 'upper tail' of the size distribution, assuming that the same breeding procedure will be practiced in all generations. The proportions *f* = 0.2 and *m* = 0.8 were used in the estimates.

The inbreeding effective population size was estimated as (Lande & Barrowclough, 1987)

$$N_{ec(i)} = \frac{4N_{ef(i)}N_{em(i)}}{N_{ef(i)} + N_{em(i)}},$$

where $N_{ef(i)}$ and $N_{em(i)}$ are the inbreeding effective population sizes in females and males, respectively, calculated as

$$N_{ef(i)} = \frac{N_f \bar{k}_f - 1}{\bar{k}_f + \frac{\sigma^2_{kf}}{\bar{k}_f} - 1}$$

and

$$N_{em(i)} = \frac{N_m \bar{k}_m - 1}{\bar{k}_m + \frac{\sigma^2_{km}}{\bar{k}_m} - 1} .$$

Note that for both formulae, estimates of effective population size are applicable to a situation in which both parents and offspring are enumerated at the same stage of development, preferably at sexual maturity. Crow and Morton (1955) suggested that the mean number of offspring should be adjusted to two before the effective size is calculated. They reasoned that in any population whose the size is not expanding or decreasing at a very rapid rate, the average number of offspring at sexual maturity will not be very far from two.

The progeny number used for calculating effective population size (both inbreeding- and variance effective number) were adjusted following Hedrick *et al.* (1995). In this procedure, assuming that survival of individuals was at random, the population was constant in size, and generations were discontinuous. The number of progeny were multiplied by the survival to spawning for males (s_m) and females (s_f), where $s_m = 2N_m/N_p$ and by $s_f = 2N_f/N_p$. N_p is the total number of progeny. The adjustment was made so that the mean number of progeny per parent equals two ($\bar{k}_m = \bar{k}_f = 2$).

(c) *Genetic parameter estimates:*

Heritability and variance components (additive, residuals and phenotypic) were estimated on the length and weight data obtained from random samples of each station

stock estimated using a derivative-free restricted maximum likelihood (DFREML) algorithm (Graser *et al.*, 1987) for an Animal Model incorporating all available pedigree information. For single traits and single records, the animal model is given as

$$y = X\underset{\sim}{\beta} + Za + e \qquad [1]$$

where

y is an N x 1 vector of single observations,

$\underset{\sim}{\beta}$ is a $p \times 1$ vector of unknown fixed effects,

a is a q x 1 vector of additive genetic effects,

e is an N x 1 vector of residuals, and

X and *Z* are known incidence matrices that assign the various effects to *y*.

Without selection, *a* and *e* have null mean and

$$\text{var}\begin{bmatrix} a \\ e \\ y \end{bmatrix} = \begin{bmatrix} A\sigma_a^2 & 0 & \sigma_a^2 AZ' \\ 0 & R & R \\ ZA\sigma_a^2 & R & \sigma_e^2 V \end{bmatrix} \qquad [2]$$

with *A* being the numerator relationship matrix, $R = I\sigma_e^2$, $V = (ZAZ'r + I)$ with $r = \sigma_a^2/\sigma_e^2 = h^2/(1-h^2)$, and *I* is an identity matrix.

A DFREML algorithm involves searching for the value of *r* that maximize the partial log likelihood function (Searle, 1979):

$$L = -\frac{1}{2}[(N - rank(X) - q)\log\hat{\sigma}_e^2 + \log|C| + q\log\hat{\sigma}_a^2 + y'Py/\hat{\sigma}_e^2], \qquad [3]$$

where

N is the number of observations (animals),

rank(X) is the number of fixed effects or the number of non-zero pivots (diagonal elements) of *C* minus *q*,

$$P = V^{-1} - V^{-1}X(X'V^{-1}X)^{-}X'V^{-1}$$

C is the coefficient matrix in the animal model [1] and defined as submatrix

$$\begin{bmatrix} X'X & X'Z \\ Z'X & Z'Z + 1/rA^{-1} \end{bmatrix}$$

where A^{-1} is the inverse of a numerator relationship matrix and is obtained by the method described by Famula (1991).

Log |C| and *y'Py* are simultaneously obtained during the absorption of the animal model equation by Gaussian elimination procedure as described by Smith and Graser (1986).

For a fixed value of *r*, σ_e^2 and σ_a^2 can be estimated as

$$\hat{\sigma}_e^2 = \frac{y'Py}{N - rank(X)} \qquad [4]$$

and

$$\hat{\sigma}_a^2 = r\hat{\sigma}_e^2 \qquad [5]$$

To obtain the REML estimate of *r*, three *r*'s (prior values) about the expected value were chosen based upon the prior knowledge of heritability of character to be estimated. Each prior *r* was used to calculate the log likelihood function *L* of [3]. Next, the prior *r* that yielded the maximum *L* and its two new adjacent values were used to calculate new *L* from equation [3]. The iterations were repeated until maximum *L* is obtained. The value of *r* that yielded the maximum *L*, thus the REML estimate, was used to calculate $\hat{\sigma}_e^2$ and $\hat{\sigma}_a^2$ using equations [4] and [5], respectively. Heritability can be estimated by $h^2 = \hat{\sigma}_a^2/\hat{\sigma}_P^2$, where $\hat{\sigma}_P^2 = \hat{\sigma}_a^2 + \hat{\sigma}_e^2$.

The REML estimates were carried out using the interactive matrix language (IML) procedure of the Statistical Analysis System computer program (SAS Institute Inc., 1989)

running on IBM RS/6000 AIX (version 4.14). The REML algorithm is listed in Appendix 1.

RESULTS and DISCUSSION

Parental identification

The large number of microsatellite loci and their high variability make them potentially important tool for parentage and kinship studies. The major concerns about using microsatellites in such studies are (1) the inheritance pattern and (2) mutation rate of microsatellite regions. Although many studies have reported Mendelian inheritance of microsatellite alleles (*e.g.*, Tautz, 1989; García de León, *et al.*, 1995; Herbinger *et al.*, 1995; Naciri *et al.*, 1995), it is commonly known that PCR amplification of microsatellites can generate 'null alleles' or non-amplifying alleles in human and other mammals studies (*e.g.* Callen *et al.*, 1993; Koorey *et al.*, 1993; Pemberton *et al.*, 1995). Non-amplifying alleles can be detected through mismatches between known parent-offspring thus showing non-Mendelian inheritance, and by significant deviations from Hardy-Weinberg equilibrium (Pemberton *et al.*, 1995). None of microsatellites used in this study appeared to have non-amplifying alleles. Mendelian inheritance of the five microsatellite markers has been verified in the separate experiment (*see* Chapter 2). In addition, we were able to detect all possible alleles (*i.e.*, 2-bp size increment) covering the size range of the four microsatellites (*Pgon-17*, *Pgon-69*, *Pgon-75* and *Pgon-79*) in the population studies (Chapter 3 and 4). Therefore, it is unlikely that null alleles would be a problem in these markers.

Mutation leads to false parentage and kinship identification as it generates novel alleles that are not present in the parents. Mutation rate at the microsatellite regions has been estimated to be relatively high (10^{-2} to 10^{-5}, Weber & Wong, 1993). Mutation may pose a serious problem in assessing an individual's kinship when its parents are unknown.

Using one to five microsatellite markers enabled the identification of most offspring (99.89%) to parental pairs. Out of 1,800 samples only two individuals cannot be identified. One sample failed to amplify and the other fish possessed a genotype profile that cannot be matched to any parental couples. False identification can arise due to mutation, non-amplifying alleles (null alleles), or scoring mistakes in the offspring. Re-amplification of the individual verified its genotypes. Mutation was, however, excluded from the probable explanation of the latter since simultaneous mutations at all four of the markers would be required. Its extremely large size (12.3 cm in length and 64.4 g in weight) from the group average (Phichit largest group, Table 5.1.2) suggested possible contamination from other sources.

Reproductive success and progeny performance

Summaries of the number of progeny contributed in each sample group from each dam and sire and their size-at-3 month of age are shown in Table 5.1.1 - 5.1.3. Results of analysis of variance for each variable in each station stock are shown in Table 5.2.

There were large and highly significant differences in the number of progeny contributed by different dams in all populations (Table 5.1 and 5.2). The observed number of progeny from each female were consistent in both random and large-size

groups, *i.e.*, the female that contribute a large number of progeny in the random sample also contributed a large number of progeny in the large-size sample group, and *vice versa*. The number of progeny sired by each male within dams appeared to be very similar for most families. Although some differences were observed in a few cases there was no obvious pattern, and the differences are suspected to be the result of sampling error. Little differences in male fertilizing success and subsequent survival of progeny was also reported in rainbow trout by Herbinger *et al.* (1995). They also reported significant differences in survival of progeny among females. Large variations in progeny size are usually related to the fertility of females. In this experiment, however, the number of eggs was approximately equal in all breeding groups, and yet large significant differences in number of progeny were observed in all studied populations. This suggested that other factors may have strong effect on the survivability of progeny among females. Gall (1974) and Pitman (1979) reported poor survival in small rainbow trout eggs. However, Herbinger *et al.* (1995), Springate & Bromage (1985) and Springate *et al.* (1985) reported no correlation between initial egg sizes and the survivability of offspring. Genetic effects are other possible factors that can affect survivability of offspring (Herbinger *et al.*, 1995).

Large significant differences among dams for their progeny growth were observed in both random and large-size sample groups in all populations, except the large group of Phitsanulok population (Table 5.2). Maternal effects are believed to play an important role in progeny performance. Correlation between egg sizes and either offspring survival or size of first feeding fry were reported in many studies (Gall, 1974; Chevassus & Blanc, 1979; Pitman,1979; Springate & Bromage, 1985; Springate *et al.*, 1985; Ferguson *et al.*, 1995). Here strong positive significant correlations (r^2 = 0.416 to

0.997) were observed between progeny growth and progeny survivability of the dams in all random groups. This finding was similar to the study of Herbinger *et al.* (1995) in rainbow trout. They concluded that genetic effect is likely to be the result of the differences among dams for their one-year offspring growth and survivability since the maternal effects seem to disappear or be reduced within a few months of growth (Kanis *et al.*, 1976; Chevassus & Blanc, 1979; Springate & Bromage, 1985; Springate *et al.*, 1985, Bromage *et al.*, 1992). In this study, dam effects for both survivability and growth performance of progeny were very strong after 3 months of growth. The generation period of the *P. gonionotus* under aquaculture condition is approximately 5-8 months. The results seemed to support Herbinger's conclusion that true genetic effects of dams can account, in part, for differences in survivability and growth of their progeny.

The maternal half-sib mating design allows the effects of males to be tested efficiently as maternal factors, such as egg size were controlled for in the design. Significant differences among sires within dam group for their growth were observed in two populations (PL and TK) in the random sample group and only one population (PL) in large-size sample group (Table 5.2). These findings suggested the significant male contribution to their progeny growth is also due partially to genetic effects. Unlike the females, no obvious relationship between progeny survivability and growth of their progeny was observed.

Effective population sizes

Effective population size is the average number of individuals in a population which actually contribute genes to succeeding generations. The effective population can be used to the calculate rate of inbreeding (Falconer, 1981) or to assess evolutionary

effects of stochastic forces on small populations (Wright, 1931, 1938). The effective size can be different from the adult census due to fluctuating population size, migration, sex ratio, variance in progeny number, and whether generations are continuous or discrete (Ewens, 1979).

Single locus microsatellite DNA fingerprinting allows us to assess the breeding structure of the population, and therefore its effective size can be accurately estimated. In this study, two methods of estimating effective size, *i.e.*, inbreeding effective number and variance effective number, were calculated and compared between groups of random samples and of the large 'upper tail' samples. Inbreeding effective number is appropriate when one wants to assess the increase of homozygosity due to random drift, while the variance effective number is appropriate when the concern is the amount of gene frequency drift or the increase in variance among subgroups (Crow & Denniston, 1988). The inbreeding and variance effective population sizes for the large 'upper tail' and random sample groups are given in Table 5.3. The inbreeding and variance effective sizes were similar with the variance effective number slightly higher for both sample groups in all three hatchery stocks.

The effective number of the random group was approximately 70% to 138% and 45% to 99% larger than those of the large 'upper tail' group for the inbreeding and variance effective population sizes, respectively. The smallest effective numbers were observed for both sample groups in Phichit breeding set due to only one female that clearly dominated in the number of progeny.

Maintaining genetic diversity and keeping inbreeding to a minimum are an important concern in the hatchery-enhancement program. A low effective size could lead to increased inbreeding that may lower fitness (Hedrick *et al.*, 1995). The rate of

inbreeding (ΔF) and the rate of loss of heterozygosity over t generations (H_t) are the reciprocal function of the effective population size and can be estimated as follow:

$$\Delta F = \frac{1}{2N_e} \qquad \text{(Falconer, 1981)}$$

$$H_t = \left(1 - \frac{1}{2N_e}\right)^t \qquad \text{(Ely, } et\ al.\text{, 1991).}$$

The effective population size can be considerably less than the number of mature adults due to (1) unequal numbers of males and females, (2) temporal variation in population number, and (3) greater than binomial or Poisson variability in the number of progeny per parent (Crow & Denniston, 1988). It has been reported that if individuals are chosen equally from all families, *i.e.*, two individuals from each pair of parents, and breeding with equal numbers of males and females, then the effective number can become nearly double of the adult census (N_e= *2N-1*, Falconer, 1981). However, under the large operating hatchery, where mass spawning is usually practiced, it is not possible to employ such methods. In Thailand, broodstock are usually replenished by the samples from the large 'upper tail' group of each mass spawning parents (personal communication). The result in this present study indicated that such a practice may lead to an increase in detrimental genetic effects. For instance, the rate of inbreeding were estimated to be about 70% to 138% higher when replenishment occurs from large individuals as compared to when they are chosen randomly. This seemed to raise the question whether genetic improvement of stock by mass selection should be routinely practiced in all hatcheries. In my opinion, the objectives in each hatchery for producing fish seed must be clarified so that the stock can be proper managed genetically. For the hatcheries where fish are being produced for release into natural water, maintaining both

genetic variability and minimal inbreeding should be of important interest and, therefore, selection must be avoided. The results here indicated that replenishing broodstock by randomly choosing from their offspring would ensure a larger effective population number than using the large 'upper tail' individuals.

Genetic parameter estimates

Heritability of a quantitative character is important as its value allows animal breeders (1) to choose an appropriate selection method and (2) to predict their success in changing the characteristic of the population when the selection is employed. Heritability is defined as the ratio of additive genetic variance to phenotypic variance ($h^2 = \sigma_a^2/\sigma_P^2$, Falconer, 1981). The additive or breeding values of individuals determines their influence on the next generation, but only their phenotypes can be directly measured. Heritability serves as the parameter relating the two values. In fish, heritability is usually estimated by sib analysis or is obtained after selection has been applied (*i.e.*, realized heritability). Both methods require information from individuals of half- and full-sub families (sib analysis), or from control and selected groups (realized heritability). Since individual tagging of fish at the fry and fingerling stages is difficult to achieve, individuals from different full-sibs or groups must be kept separate until they can be tagged. In many experiments, these fish were reared separately throughout the growing period. By rearing the same family or group together, we do not only reduce the variance within family or group, *i.e.*, fish grown together tend to resemble to each other because they have experienced the same environment (common environmental variance), but also increase variance between families or groups (replicate variances of unpredictable magnitudes and unknown distributions, Uraiwan and Doyle, 1986) as they

have experienced different environments. In addition, fish are known to interact with each other and this usually results in differences of growth. Shoot-carp is a good example of competitive interaction between individuals resulting in a few individuals out-growing others. Competition can reduce resemblance between relatives when they compete for limited resources, and therefore contributes an additional source of variation within families (Falconer, 1981). These additional variances often make the heritability estimate less reliable, or sometimes, meaningless. Falconer (1981) suggested that common-environment variance can be eliminated by dividing families into two or more groups so the variance component can be measured and deduced. By doing this, however, we further introduce another "replicate variance" to the estimates.

Existence of large number of alleles per locus in many microsatellites of various fish species (Ambali, 1996; Brooker *et al.*, 1994; and Kamonrat, this study) makes parentage identification in large family groups possible, and therefore allows family group to be reared together from birth. This increases the reliability of the heritability estimate as well as simplify experimental designs. It is shown here that only a single pond is needed for grow-out of individuals of half- and full-sib families in the nested mating design for the estimation of heritability. Since all fish were reared together from birth, both common-environment and "replicate" variances can be eliminated as well as allowing all individuals to naturally interact to each other.

A number of methods can be used in quantitative variance component estimates including the analysis of variance (ANOVA) by method 1, 2 and 3 of Henderson (1953), maximum likelihood (ML, Hartley & Rao, 1967), restricted maximum likelihood (REML, Patterson & Thompson, 1971), minimum norm quadratic unbiased estimation (MINQUE, Rao, 1970, 1971) and its variants, *etc.*, (Kennedy, 1981). Among these methods, ML

and REML possess several attractive properties, such as non-negativity of estimates, and can account for non-random sampling of data (Kennedy, 1981). Negative estimates occasionally observed with the ANOVA method and how to handle such situations has never been totally resolved. Here the REML method was used to estimate variance components and heritability of size (standard length and body weight) of 3 months for *P. gonionotus* in three hatchery populations. The REML method is a modification of ML to handle the unknown properties of fixed effects that lead to biased results in ML estimators (Patterson & Thompson, 1971). Under REML, only the proportion of the likelihood that is invariant to the fixed effects is maximized so that the bias due to the estimation of fixed effects is removed. In a completely random model, however, both ML and REML yield the same results.

Heritability estimates are considered low to moderate for the three hatchery stocks and range from 0.290 to 0.523 and 0.193 to 0.421 for standard length and body weight, respectively (Table 5.6). The highest heritability estimate was obtained for the Phitsanulok stock and the lowest was obtained for the Phichit stock. The highest variance component estimates were, however, obtained for the Tak stock. It should be noted that variance components and heritability estimates here are based on maternal half sibs. Maternal effects are important in many animals, particularly in mammals, where mothers take care of their young. In such a case, maternal effects often contribute an environmental component to the covariance of offspring with mothers, and to the covariance of maternal half sibs (Falconer, 1981), therefore, inflating the estimates. In fish, however, evidence suggests that maternal effects are less important (see above), and therefore maternal half-sib heritability estimate may be more reliable than in mammals.

Previous heritability estimates for traits related to growth in fish were varied, many were not different from zero, making selective breeding in fish questionable despite their advantages over land animals in terms of great fecundity, high genetic variability (heterozygosity), *etc.* (Wilkins, 1981). The results here indicated that heritability of growth trait in fish may actually be higher than previously observed providing various environmental variances are eliminated from the estimates by common rearing. Moderate to high paternal half-sib heritability estimates for body weight (h^2= 0.61) and standard length (h^2= 0.48) were also obtained in Nile tilapia (*Oreochromis niloticus*) under communal rearing (Velasco *et al.*, 1995).

Selective breeding program of Puntius gonionotus

Relatively high heritability of growth traits estimated in all three *P. gonionotus* stocks indicating that selective breeding program for growth improvement of this species should result in a good progress. It should be noted, however, that the number of effective breeding size is greatly reduced (70% to 138%) when individuals were to selected from the largest "upper tail" of size distribution as compared to random chosen individuals. Therefore the simple means selection strategies, such as individual or mass selection in which the decision is based upon individuals "best" phenotypic values regardless of their relationships (Falconer, 1981), is very likely result in rapid inbreeding in a population.

Within-family selection considers each family as a temporary sub-population and selection is applied independently within each family whereby individuals that exceed their family mean by the greatest amount being regarded as the most desirable (Falconer, 1981). Because selection occurs independently in each family, inbreeding is

minimized under this selection procedure. Furthermore, if two members of every family are selected to replace the parents, then every family contributes equally to the parents of the next generation which gives the effective population size twice the actual (see above). Within-family selection has been suggested to be used when the heritability of character under selection is small and a large component of variance is due to common environment as selection within families would eliminate this large non-genetic portion from the variation operated on by selection (Falconer, 1981). Generally, the procedure requires that members of a family can be recognized at the time of selection. Unless the families are marked, each family must be grown in a single pond or cage. Consequently, several ponds or cages are required in order to keep the rate of inbreeding below a certain value. Microsatellites can be used to establish pedigrees in a population even when the parents are unknown (Herbinger *et al.*, 1996) allowing all families to be grown in a single pond. Doyle and Herbinger (1995) demonstrated that within-family selection based on microsatellite DNA fingerprinting (called "walk-back") can be done in a commercial farm without interference with the normal routine. However, their estimate cost effectiveness of the procedure is still 30 to 100 fold higher than classical combined selection using physical tagging procedures. Microsatellite genotyping cost has greatly reduced during the past years (D. Cook *pers comm.*) would enable the use of microsatellite in such selection procedure.

Correlation between microsatellite diversity and genetic variances

The relationship between additive genetic variance (σ_a^2), total phenotypic variance (σ_P^2) and genetic diversity of the populations was assessed. Genetic diversity estimates at 4 microsatellite loci in each hatchery stock of the three fisheries station were

previously obtained from samples of the station broodstocks (*see* Chapter 4). There were indications of a strong negative relationship between additive genetic variance and the effective number of alleles per locus, but no relationship between additive genetic variance and the heterozygosity (Fig. 5.1). Similar trends were observed for the relationship between total phenotypic variance and effective number of alleles per locus or observed heterozygosity (Fig. 5.2).

In small populations, the consequence of genetic drift, inbreeding and restricted gene flow is known to decrease genetic variation. A smaller number of variable loci and a smaller number of alleles have been reported in small populations (*e.g.*, Hamrick *et al.*, 1979; Levin *et al.*, 1979; Schmidtke & Engel, 1980; Moran & Hopper, 1983; Karron, 1987; Van-Treuren *et al.*, 1991). However, no correlation between gene diversity (heterozygosity) and the size of population has been reported (Varvio-Aho, 1981; Van-Treuren *et al.*, 1991). Van-Treuren *et al.* (1991) reasoned that genetic drift can make rare alleles reach high frequencies and therefore inflate gene diversity in small populations. This may explain, in part, the non-relationship between (quantitative) variance components and the observed heterozygosity in this study. The apparent negative relationship between the additive genetic variance, total phenotypic variance and the effective number of alleles per locus are, however, unexpected. Since individuals of different genotypes would be expected to react differently under the same environment, the population having many effective alleles, *i.e.*, consisting of many genotypes, should also exhibit heterogeneity in fitness characters. This study only dealt with 3 populations and therefore the relationship might simply be the product of chance. Further research needs to be done on more populations to see whether the relationship is reproducible.

Molecular marker variation, such as allozymes and microsatellite DNA is not, by assumption, influenced by environments. Analyses based on these data are therefore much more powerful than those based on phenotypic characters, such as body length and weight, for detection of genetic variation between and within populations (Ryman, 1983). However, it is phenotypic characters that are of economic importance. Molecular genetics offers a variety of tools that can be used to examine the genetic structure of population both at the genome level (*e.g.*, DNA sequences, DNA fingerprinting of mini- and microsatellites) and at the level of gene products (*e.g.*, protein electrophoresis). Direct used of this information must be approached with caution. Molecular techniques randomly detect genetic variation based on surveys of protein or DNA at particular regions of the genome. To assume that the levels of variation detected at marker loci directly reflect the level of variation that influences future adaptation or individual fitness is, therefore, questionable (Miligan *et al.*, 1994). Many reports suggested that the relationship may be less straightforward then commonly assumed (*e.g.*, Houle, 1989; Booth *et al.*, 1990; Gaffney, 1990; Hutchings & Ferguson, 1992; Leberg, 1993; Pogson & Zouros, 1994; Ferguson *et al.*, 1995). The existence of a relationship cannot be, however, ruled out as the nature of their variation is not fully understood. The unclear relationship between degree of variation in molecular markers and individual fitness makes the task difficult for genetic based management of populations and further studies are required.

CONCLUSION

It is demonstrated in this study that kinships in a large communal rearing (four half-sibs and 16 full-sibs families) of *P. gonionotus* can be identified using only a few microsatellites markers. In addition to successfully identifying parentage of all sampled individuals in all three populations, microsatellite markers distinguished possible contaminating or 'jumper' fish from other sources. This result demonstrated the significant power of microsatellites as a tool for parentage and kinship studies. Ability of identify individuals from large families under communal rearing allows researchers to carry out more efficient experiments in a limited space and/or under natural conditions. The information such as the effective population size, inbreeding rates, differences in offspring survivability and the growth of sires and dams can now be easily assessed. Such informations will assist hatchery managers develop more effective breeding plans.

Here it was shown that genetic study of relatively complicated design can be carried out in a single pond. The quantitative variance components and heritability estimates derived from this approach are believed to be accurate and reliable because several unwanted sources of variance (*i.e.*, common-environmental and replicated variances) are eliminated. Interaction between individuals is allowed to occur naturally which increases the biological relevance of the variance estimates. The estimates of h^2 are reasonably high (0.193 to 0.523) and encouraging for selection programs. The fact that microsatellites can be used to establish pedigrees in a population even when the parents are unknown (Herbinger *et al.*, 1996) will allow the estimate of variance components simply by random sampling of progeny produced by natural mass spawning, such as that is practiced in *P. gonionotus* production in Thailand.

Table 5.1 Summary for Phitsanulok stock. N_{OFF} is the number of progeny, LT is standard length (in cm), and WT is weight (in grams). Superscript letters (a,b,c) indicate pair-wise (post hoc) comparisons, where different letters between pair of dams indicated significant differences (P <0.05) and the same letter indicates non-significance.

		RANDOM GROUP			LARGE GROUP		
DAM	SIRE	N_{OFF}	LT±SE	WT±SE	N_{OFF}	LT±SE	WT±SE
1	1	9	4.178±0.700	2.522±1.959	2	6.700±0.700	11.350±3.650
1	2	40	5.310±1.077	5.258±3.171	28	7.482±0.162	15.996±1.247
1	3	9	3.744±0.207	1.533±0.212	2	10.300±2.000	40.000±20.000
1	4	13	5.200±1.248	5.531±3.771	5	6.840±0.273	12.100±2.056
DAM-1		71^{ab}	$4.608±0.016^{a}$	$3.711±0.046^{a}$	37^{a}	$7.831±0.032^{a}$	$19.862±0.261^{a}$
2	1	31	5.997±1.000	7.803±3.465	69	7.719±0.118	18.481±0.912
2	2	26	5.312±1.232	5.419±3.201	26	7.815±0.218	18.585±1.772
2	3	35	5.226±1.231	5.589±3.722	26	7.142±0.147	14.254±1.058
2	4	46	5.970±1.180	7.696±3.823	94	7.638±0.087	17.224±0.650
DAM-2		138^{a}	$5.626±0.009^{b}$	$6.627±0.027^{b}$	215^{b}	$7.579±0.004^{a}$	$17.136±0.033^{a}$
3	1	28	4.721±1.146	3.975±3.271	15	7.887±0.338	19.673±2.619
3	2	7	4.071±0.509	2.286±0.815	1	7.500±0.000	15.000±0.000
3	3	8	4.313±0.892	2.850±2.207	7	8.014±0.430	20.871±2.969
3	4	20	5.085±1.241	5.235±3.913	19	7.774±0.214	18.811±1.780
DAM-3		63^{ab}	$4.548±0.018^{ac}$	$3.587±0.053^{a}$	42^{ab}	$7.794±0.030^{a}$	$18.589±0.202^{a}$
4	1	5	3.900±0.367	1.800±0.418	2	7.650±0.350	17.500±2.500
4	2	8	4.300±1.038	3.163±2.714	2	8.000±1.200	19.900±10.100
4	3	9	4.000±0.532	2.122±1.067	0	--	--
4	4	6	4.917±1.789	5.367±5.688	2	7.200±0.200	12.700±0.300
DAM-4		28^{b}	$4.279±0.038^{c}$	$3.113±0.113^{a}$	6^{a}	$7.617±0.146^{a}$	$16.700±1.226^{a}$

Table 5.2 Summary for Phichit stock. N_{OFF} is the number of progeny, LT is standard length (in cm), and WT is weight (in grams). Superscript letters (a,b,c) indicate pair-wise (post hoc) comparisons, where different letters between pair of dams indicated significant differences (P <0.05) and the same letter indicates non-significance.

		RANDOM GROUP			LARGE GROUP		
DAM	SIRE	N_{OFF}	LT±SE	WT±SE	N_{OFF}	LT±SE	WT±SE
1	1	99	4.501±0.108	3.097±0.241	132	6.172±0.070	7.514±0.277
1	2	27	4.704±0.205	3.444±0.426	49	6.320±0.117	7.851±0.453
1	3	31	4.168±0.211	2.552±0.389	22	5.855±0.177	6.205±0.557
1	4	57	4.254±0.151	2.795±0.335	78	6.388±0.082	8.127±0.298
DAM-1		214[a]	4.407±0.005[a]	2.972±0.011[a]	281[a]	6.184±0.003[a]	7.425±0.011[a]
2	1	2	3.600±0.200	1.350±0.150	0	--	--
2	2	2	3.750±0.450	1.500±0.500	0	--	--
2	3	6	3.150±0.277	0.950±0.292	0	--	--
2	4	1	2.600±0.000	0.600±0.000	0	--	--
DAM-2		11[b]	3.275±0.057[b]	1.100±0.057[b]	0	--	--
3	1	2	2.700±0.200	0.500±0.100	0	--	--
3	2	0	--	--	0	--	--
3	3	3	2.767±0.067	0.567±0.067	0	--	--
3	4	1	2.300±0.000	0.200±0.000	0	--	--
DAM-3		6[b]	2.589±0.039[b]	0.422±0.029[b]	0	--	--
4	1	24	3.533±0.217	1.625±0.321	7	5.800±0.269	6.157±0.901
4	2	1	2.500±0.000	0.500±0.000	0	--	--
4	3	28	3.393±0.149	1.264±0.201	5	5.640±0.319	5.460±0.964
4	4	16	3.556±0.249	1.544±0.358	6	5.500±0.344	5.100±1.024
DAM-4		69[ab]	3.246±0.014[b]	1.233±0.019[b]	18[b]	5.647±0.040[b]	5.572±0.126[b]

Table 5.3 Summary for Tak stock. N_{OFF} is the number of progeny, LT is standard length (in cm), and WT is weight (in grams). Superscript letters (a,b,c) indicate pair-wise (post hoc) comparisons, where different letters between pair of dams indicated significant differences (P <0.05) and the same letter indicates non-significance.

		RANDOM GROUP			LARGE GROUP		
DAM	SIRE	N_{OFF}	LT±SE	WT±SE	N_{OFF}	LT±SE	WT±SE
1	1	11	5.827±0.364	7.345±1.689	7	8.243±0.297	19.329±1.924
1	2	14	5.036±0.384	5.250±1.068	4	7.150±0.206	12.100±1.217
1	3	32	5.259±0.227	5.750±0.655	21	7.476±0.186	14.600±1.294
1	4	10	5.800±0.647	8.650±3.222	12	7.883±0.360	17.483±2.421
ALL		67^{a}	$5.481±0.021^{a}$	$6.749±0.082^{a}$	44^{ab}	$7.688±0.022^{a}$	$15.878±0.149^{a}$
2	1	2	8.900±0.900	25.450±8.750	11	8.364±0.237	20.527±1.955
2	2	47	6.547±0.274	11.534±1.278	90	8.153±0.094	19.091±0.662
2	3	22	7.000±0.282	12.573±1.340	37	7.986±0.141	17.914±0.977
2	4	32	6.306±0.308	10.341±1.455	37	7.959±0.138	17.608±0.967
ALL		103^{a}	$7.188±0.017^{b}$	$14.975±0.081^{b}$	175^{a}	$8.116±0.005^{b}$	$18.785±0.035^{b}$
3	1	23	5.426±0.241	6.148±0.757	20	7.635±0.181	15.070±0.865
3	2	42	5.643±0.223	7.436±0.949	32	7.772±0.142	17.122±0.934
3	3	32	4.619±0.210	3.941±0.575	12	7.650±0.178	16.258±1.236
3	4	14	5.000±0.305	4.950±0.849	6	8.417±0.312	21.100±2.521
ALL		111^{a}	$5.172±0.012^{a}$	$5.619±0.043^{a}$	70^{ab}	$7.869±0.011^{a}$	$17.388±0.071^{a}$
4	1	6	4.717±0.575	4.350±1.594	4	7.950±0.357	17.575±2.358
4	2	8	4.338±0.332	3.050±0.721	2	8.000±0.000	17.150±0.150
4	3	1	5.400±0.000	5.700±0.000	2	8.450±0.450	21.500±3.700
4	4	3	5.100±0.379	4.400±1.039	3	8.667±0.406	23.200±3.345
ALL		18^{a}	$4.889±0.059^{a}$	$4.375±0.149^{a}$	11^{b}	$8.267±0.058^{a}$	$19.856±0.441^{a}$

Table 5.4 Analysis of variance of standard length, body weight, and number of progeny (N_{OFF}) for each sampling group in Phisanulok (PL), Phichit (PC), and Tak (TK) stocks.

Group	Variable	Source	MS	F-RATIO	P-value
Random-PL	Length	DAM	0.4035	23.6278	<0.0001
		SIRE{DAM}	0.0688	3.9159	<0.0001
	Weight	DAM	9.1588	25.2702	<0.0001
		SIRE{DAM}	1.5487	4.2732	<0.0001
	N_{OFF}	DAM	0.0098	6.2969	0.0082
Large-PL	Length	DAM	0.0005	0.2312	0.8746
		SIRE{DAM}	0.0043	2.1596	0.0167
	Weight	DAM	0.0963	0.1956	0.8994
		SIRE{DAM}	0.8424	1.7100	0.0707
	N_{OFF}	DAM	22.2205	6.7398	0.0091
Random-PC	Length	DAM	1.0175	14.2006	<0.0001
		SIRE{DAM}	0.0660	0.9212	0.5202
	Weight	DAM	9.7322	13.6252	<0.0001
		SIRE{DAM}	0.6000	0.8401	0.6000
	N_{OFF}	DAM	26.4849	10.7819	0.0013
Large-PC	Length	DAM	2.5275	7.6142	0.0062
		SIRE{DAM}	0.6760	2.0366	0.0735
	Weight	DAM	4.7399	7.4715	0.0067
		SIRE{DAM}	1.3238	2.0867	0.0671
	N_{OFF}	DAM	52.9767	10.6366	0.0224
Random-TK	Length	DAM	0.5713	11.4121	<0.0001
		SIRE{DAM}	0.0985	1.9678	0.0271
	Weight	DAM	4.9952	11.5237	<0.0001
		SIRE{DAM}	0.9260	2.1361	0.0150
	N_{OFF}	DAM	7.6994	3.4967	0.0498
Large-TK	Length	DAM	0.0039	3.0362	<0.0295
		SIRE{DAM}	0.0017	1.2849	0.2266
	Weight	DAM	0.2413	3.2557	0.0221
		SIRE{DAM}	0.1082	1.4596	0.1389
	N_{OFF}	DAM	14.7500	6.1540	0.0089

Table 5.5 Effective population sizes for the largest 'upper tail' and random sample groups in the three hatchery stocks as estimated by expressions for inbreeding and variance effective population sizes. Number of individuals mating are in parentheses Progeny number were adjusted (detailed in text).

Parameter	Phisanulok Large	Phisanulok Random	Phichit Large	Phichit Random	Tak Large	Tak Random
Inbreeding effective population size						
Male: $N_{em(i)}$ (16)	6.205	15.071	3.863	6.155	8.006	13.959
Female: $N_{ef(i)}$ (4)	1.689	3.997	0.888	1.611	2.534	4.259
Total: $N_{ec(i)}$ (20)	5.310	12.637	2.874	5.108	7.698	13.054
Variance effective population size						
$N_{ec(v)}$ (20)	6.979	13.205	3.427	6.836	9.004	13.084

Table 5.6 Variance components and heritability estimates for standard length and body weight of 3 month old *Puntius gonionotus* in the three fisheries stations. σ_a^2 = additive genetic variance, σ_e^2 = residual variance = $(1-h^2)/h^2$, σ_P^2 = total phenotypic variance = $\sigma_a^2 + \sigma_e^2$, and h^2 = heritability = σ_a^2/σ_P^2.

	σ_a^2	σ_e^2	σ_P^2	h^2
Phitsanulok stock				
Standard length (cm)	0.883	0.803	1.686	0.523
Body weight (g)	6.900	9.453	16.354	0.421
Phichit stock				
Standard length (cm)	0.375	0.915	1.290	0.290
Body weight (g)	0.968	4.034	5.002	0.193
Tak stock				
Standard length (cm)	0.926	1.716	2.642	0.350
Body weight (g)	14.835	31.564	46.399	0.319

Microsatellite DNA variation at 4 loci in three fisheries station (from **Chapter 4**) .

Population	Mean no. of alleles per locus	Effective number of allele	Mean heterozygosity		F_{IS}
			Direct-count	HdyWbg expected	
Phitsanulok	12.8±3.8	7.082	0.743±0.060	0.827±0.042	0.1016
Phichit	12.3±4.4	8.152	0.801±0.063	0.830±0.053	0.0349
Tak	10.5±3.0	4.829	0.783±0.042	0.779±0.035	-0.0051

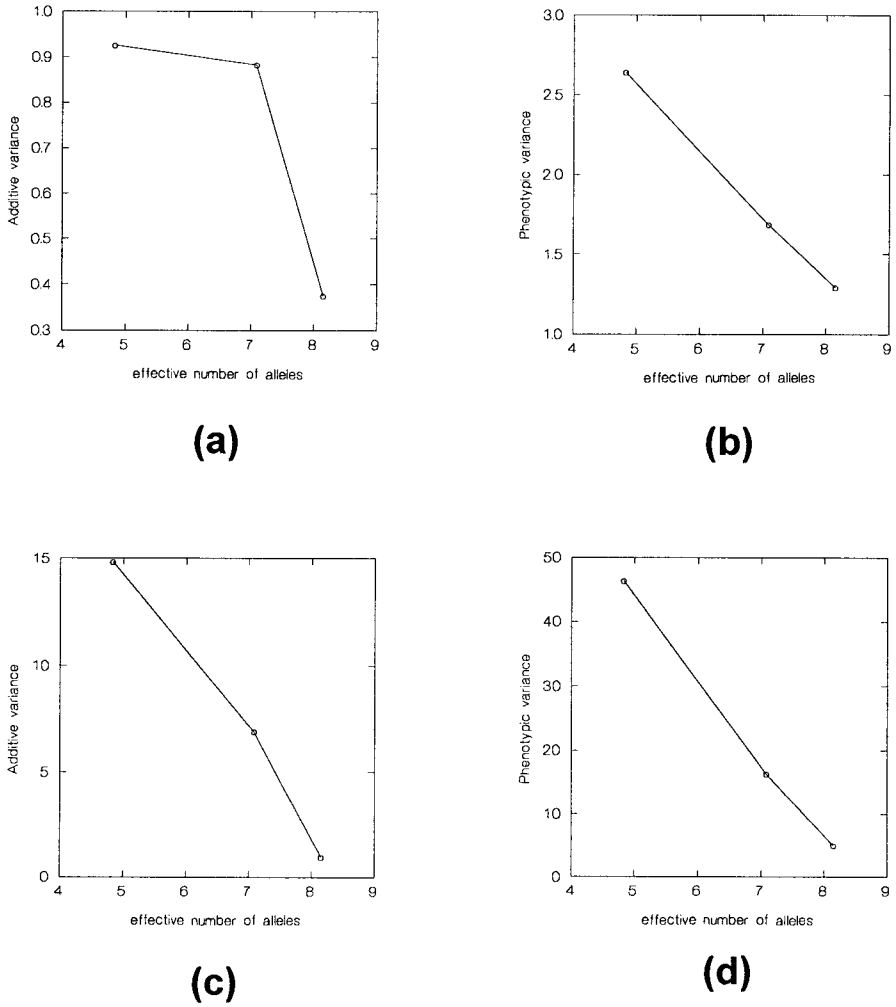


Figure 5.1 Relationship between additive, phenotypic variances [of standard length (a) and (b), and of body weight (c) and (d)] and the effective number of alleles.

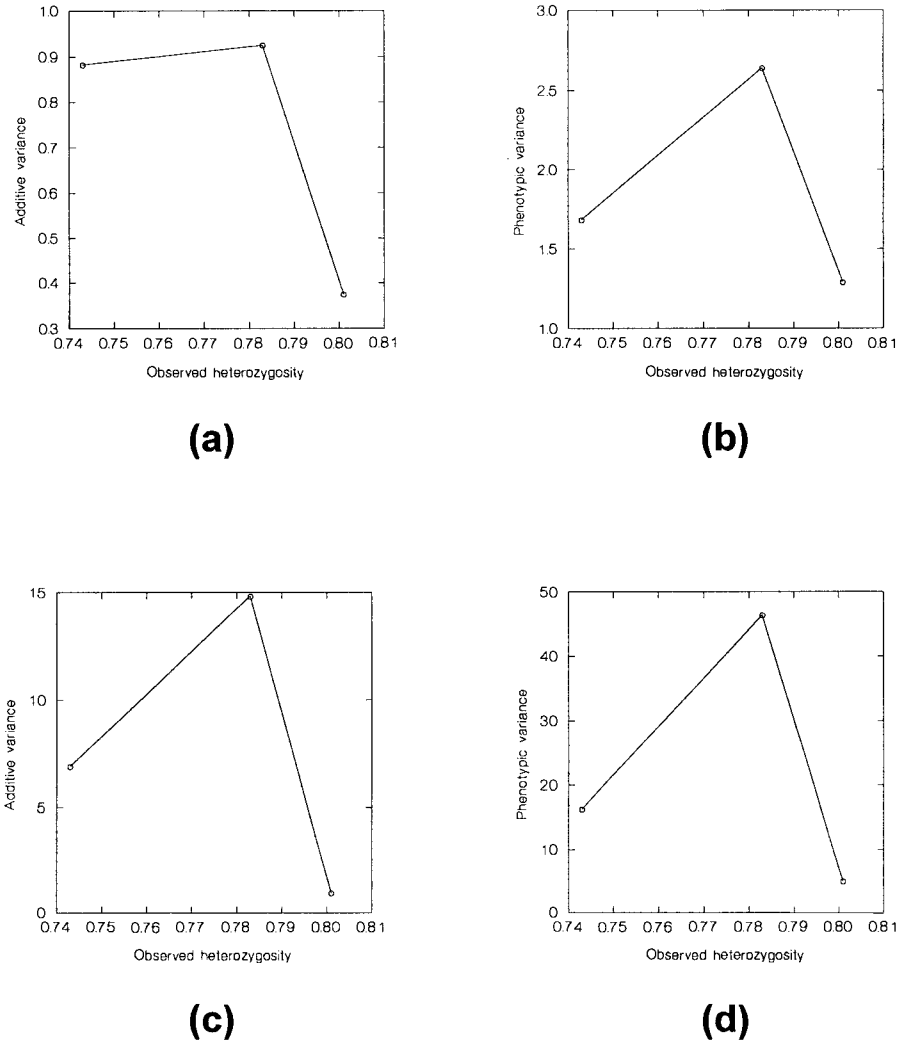


Figure 5.2 Relationship between additive, phenotypic variances [of standard length (a) and (b), and of body weight (c) and (d)] and the observed heterozygosity.

Chapter 6

General Conclusions

Knowledge of the genetic structure of fish stocks or populations is increasingly required for both fisheries and aquaculture management. For natural populations, understanding the amount and patterns of distribution of genetic variability within species will assist in the efficient use of natural resources, e.g., mixed stock fisheries management, and avoid an unwanted reduction of genetic variation due to unwise management. Information on both spatial distribution and temporal changes of harvested populations helps determine the optimal management strategy for both fisheries and conservation. In aquaculture, genetic variation is the basic resource for any successful animal breeding program. Information on genetic variation within hatchery stocks indicates the level of success in their management and also the status of their broodstock. Genetic diversity between stocks is also critical when one considers hatcheries as *gene banks* for conserving genetic resources.

Molecular approaches, such as protein electrophoresis, have long provided effective tools for fish population studies and fishery management (Utter, 1991). However, the more recent developed microsatellite DNA markers have added new dimensions in the field of fisheries and aquaculture where many populations are recently separated or have gone through severe bottleneck events or inbreeding, and as a consequence exhibit low variation and traditional markers cannot be used. In this thesis four microsatellites developed from the Thai silver barb (*Puntius gonionotus*) genomic library exhibited a high level of polymorphism in several *P. gonionotus* populations similar to those reported in other teleosts, such as Atlantic cod (*Gadus morhua*; Brooker *et al.*, 1994), Atlantic salmon (*Salmo salar*; Slettan *et al.*, 1993; McConnell *et al.*, 1995*a,b*), brown trout (*Salmo trutta*; Estoup *et al.*, 1993), rainbow trout (*Onchorhynchus mykiss*;

Morris *et al.*, 1996), sea bass (*Dicentrarchus labrax*; García de León *et al.*, 1995), Stickleback (*Gasterosteus acuelatus*; Rico *et al.*, 1993), tilapias (*Oreochromis niloticus* and *O. shiranus*; Ambali, 1996), and zebrafish (Goff *et al.*, 1992). These microsatellites have been successfully used in this thesis to study structure of Thai populations the *P. gonionotus*, both natural and aquacultural.

P. gonionotus is native to Thai river systems and is an economically important species for both fisheries and aquaculture industries. The species comprises more than 20% in the total inland fisheries production and ranks as the third most important species caught or cultured in Thailand. The Thai Department of Fisheries (DOF) has been restocking this species and several others for many years to increase natural production. The mixed stock analysis (MSA) of the *P. gonionotus* samples obtained from the ChaoPhaya and MaeKlong rivers indicated 75% to 96% were of hatchery populations. Although the figures may be interpreted as a success from the management point of view, genetic resources of this species may be at high risk if interbreeding between natural and hatchery fish occurs. Here the study of the natural populations sampled from the major rivers in Thailand reveals a low but significant genetic discreteness between different watersheds. In order to preserve genetic integrity at the level of the watersheds, the Thai DOF should establish hatchery policies specifically for the restocking program. I recommend that specific stocks should be established in stations that are responsible for restocking the species in the native watersheds. These stocks should be a collection of natural population within watersheds. No transferring of stocks across watersheds should be done as it is likely to alter the genetic make-up of the locally-adapted stocks.

Hatchery practices may affect genetic resources in several ways. Microsatellite analyses of several hatchery stocks in Thailand indicated both decreasing and increasing average and effective number of alleles per locus. The change appeared to be a random process, although in many occasions a decreased number of alleles was observed when a single stock was transferred to establish a new one, while an increased number of alleles occurred when a new stock created by mixing several preexisting stocks. The traditional practice among Thai fisheries stations where replenishing broodstock from the largest fish (upper tail of the size distribution) may accelerate the deleterious effects of inbreeding and the loss of alleles as compare when brood fish are chosen at random. The effective number of breeders in the offspring chosen at random was estimated at 45% to 138% larger than the estimate from the offspring selected from the upper tail of the distribution.

In Thailand, fisheries stations represent the largest number of captive stocks of *P. gonionotus*. Each station keeps its own broodstock and hatchery management is varied. Microsatellite analyses of 29 fisheries stations in the central, north and north-east region of the country revealed a spatially subdivided structure where there were clear differences between stations in the central group and the north-east group. However, the dependency of genetic and geographic distances appeared to be weak which reflected the history of stock transfers among stations whose managers have good personal relations rather than among neighborhood stations. Nevertheless, the global structure of hatchery populations of *P. gonionotus* in Thailand can be established based on their genetic distances or similarities. Maintaining the genetic integrity of hatchery populations based on their distinctness is of concern as they represent another source of genetic diversity (in addition to a natural source) of the species. It is recommended that

the Thai DOF restricts the policy regarding stock transfers or exchanges among stations according to their genetic proximity.

Aquaculture has increasingly become an important source of fish production throughout the world. In Thailand, aquaculture contributes about 300,000 tons of freshwater and marine fish. Most aquaculture fishes are only recently removed from the wild. Selective breeding of aquaculture stocks is needed to improve their production. Animal breeding theory has been applied to breeding program in fish with little progress. Experimental results in many fish species have often been disappointing due to the low apparent heritability of growth traits. It has been suspected that the heritability of growth traits in fish may be underestimated due to confounding of competitive behavior that inflates within groups (ponds) variances (R. Doyle *pers comm.*) as full sib families are reared in separate ponds. In this study, a nested mating experiment was carried out to estimate heritability and variance components of growth traits of *P. gonionotus* in three populations. Offspring from all families were reared together from egg fertilization in a communal pond. Use of one to five microsatellite markers enabled the identification of these offspring to their parents. The heritability estimated from maternal half-sib component for standard length and body weight at 3 month old ranged from 0.290 to 0.523 and 0.193 to 0.421, respectively. This finding suggested that selective breeding for growth in fish can produce considerable improvement when the environmental variances are taken into account.

Finally, three PCR primers developed from the *P. gonionotus* genomic library in this study also amplified genomic DNA of related taxa (*P. pierrei*) in which locus *Pgon-75* exhibited a fixation of alternative alleles, where the species exhibits no common alleles at a loci (Utter & Ryman, 1993). One primer, *Pgon-22*, was also successfully used to

amplified genomic DNA of common carp (*Cyprinus carpio*; D. Morris & Y. Shi, unpublished data) and 6 species of Chinese carps including bighead carp (*Aristichthy nobilis*), silver carp (*Hypophthalmichthys molitrix*), grass carp (*Ctenopharyngodon idellus*), black carp (*Mylopharyngodon piceus*), crucian carp (*Carassius auratus*), and Wuchang fish (*Megalobrama amblycephala*) (Y. Shi & A. Ball, unpublished data) which also exhibit a fixation of alternative alleles. These findings will be useful for taxonomic studies of this taxa as well as identifying their natural hybridization.

Appendix 1

SAS/IML algorithm for REML estimates of variance components. Data is the standard length of random sampled group of the Phitchit stock.

```
proc IML;
reset noprint;

/* module for computing row & col id -ihmssf  */

START IHMSSF(IROW,JCOL,NORD,CODE);

IF IROW - JCOL < 0 THEN
  CODE = -(IROW*(IROW-1))/2+JCOL+NORD*(IROW-1);
IF IROW - JCOL = 0 THEN
  CODE = -(IROW*(IROW-3))/2+NORD*(IROW-1);
IF IROW - JCOL > 0 THEN
  CODE = -(JCOL*(JCOL-1))/2+NORD*(JCOL-1)+IROW;
FINISH;

/* module to bulid A-inverse for non-inbred populations*/
/* for half-stored A-inverse (using ihmsff)                    */

START ainv(rel,a1,nanim);
do ii = 1 to nanim;
      if rel[ii,1] = 0.0 & rel[ii,2] = 0.0 then do;
            ixx = ii;
            run ihmssf(ixx,ii,nanim,code);
            a1[code] = a1[code] + 1.0;
      end;
      if rel[ii,1] ^= 0.0 & rel[ii,2] ^= 0.0 then do;
            ixx = ii;
            run ihmssf(ixx,ii,nanim,code);
            a1[code] = a1[code] + 2.0;
            run ihmssf(ii,rel[ii,1],nanim,code);
            a1[code] = a1[code] +(-1.0);
            run ihmssf(ii,rel[ii,2],nanim,code);
            a1[code] = a1[code] +(-1.0);
            run ihmssf(rel[ii,1],rel[ii,2],nanim,code);
            a1[code] = a1[code] + .5;
            ixx = rel[ii,1];
            run ihmssf(ixx,rel[ii,1],nanim,code);
            a1[code] = a1[code] + .5;
            ixx = rel[ii,2];
            run ihmssf(ixx,rel[ii,2],nanim,code);
            a1[code] = a1[code] + .5;
      end;
      if rel[ii,1] ^= 0.0 & rel[ii,2] = 0.0 then do;
```

```
            ixx = ii;
            run ihmssf(ixx,ii,nanim,code);
            a1[code] = a1[code] + (4./3.);
            run ihmssf(ii,rel[ii,1],nanim,code);
            a1[code] = a1[code] + (-2./3.);
            ixx = rel[ii,1];
            run ihmssf(ixx,rel[ii,1],nanim,code);
            a1[code] = a1[code] + (1./3.);
      end;
      if rel[ii,2] ^= 0.0 & rel[ii,1] =0.0 then do;
            ixx = ii;
            run ihmssf(ixx,ii,nanim,code);
            a1[code] = a1[code] + (4./3.);
            run ihmssf(ii,rel[ii,2],nanim,code);
            a1[code] = a1[code] + (-2./3.);
            ixx = rel[ii,2];
            run ihmssf(ixx,rel[ii,2],nanim,code);
            a1[code] = a1[code] + (1./3.);
      end;
end;
FINISH;

/* this is a dfreml program to estimate additive variances */
/* via dfreml with a maximun likelihood search                */

/* MODULE FOR EVALUATEING THE LIKELIHOOD FUNCTION L  */

START like(lslhs,f,a1,rat,neq,nanim,nrec,nf,L1,yy,estve);

if rat[1] > 0.0 then do;
junk = j(nf,nf,0);
c = lslhs + block(junk,a1*(1./rat[1]));
temp1 = c||f;
temp2 = f`||yy;
df = temp1//temp2;

/* do the gaussian elimination */
clog = 0.0;
nrank = 0;
do i = 1 to neq;
  if df[i,i] ^= 0.0 then nrank = nrank + 1;
clog = clog + log(df[i,i]);
col = df[1:neq+1,i];
dfnew = df - (1./df[i,i])*col*col`;
df = dfnew;
end;

q = nanim;
rx = nf;
estve = df[neq+1,neq+1]/(nrec-rx);
estva = rat[1]*estve;
ypy = df[neq+1,neq+1];
```

```
L1 = -.5*((nrec-nrank)*log(estve)+clog+q*log(estva)+(ypy/estve));

end;
FINISH;

/* MAIN PROGRAM - READ IN DATA, BUILD MATRICES */
/* read in A-inv */

npar = 1;          /* parameter to be estimated                */
nanim = 319;  /* number of animals including parents       */
nat = nanim*(nanim+1)/2;
nrec = 300;        /*  number of animals with record                        */
nadu = nanim - nrec;
nf = 1;
neq = nf + nanim;
ratio = j(npar,1,0);
do ratio= .1 to 1 by .3;     /* prior value, ratio=h2/(1-h2)  */

/*  read-in relationship for randomPC  */
rel={1 5,1 5,1 6,1 6,1 7,1 7,1 7,1 7,1 7,1 7,1 8,2 9,2 9,2 9,2 9,
2 9,2 9,2 9,2 9,2 9,2 9,2 9,2 9,2 9,2 9,2 9,2 9,2 9,2 9,2 9,
2 9,2 9,2 9,2 9,2 9,2 9,2 9,2 9,2 9,2 9,2 9,2 9,2 9,2 9,2 9,
2 9,2 9,2 9,2 9,2 9,2 9,2 9,2 9,2 9,2 9,2 9,2 9,2 9,2 9,2 9,
2 9,2 9,2 9,2 9,2 9,2 9,2 9,2 9,2 9,2 9,2 9,2 9,2 9,2 9,2 9,
2 9,2 9,2 9,2 9,2 9,2 9,2 9,2 9,2 9,2 9,2 9,2 9,2 9,2 9,2 9,
2 9,2 9,2 9,2 9,2 9,2 9,2 9,2 9,2 9,2 9,2 9,2 9,2 9,2 9,2 10,
2 10,2 10,2 10,2 10,2 10,2 10,2 10,2 10,2 10,2 10,2 10,2 10,
2 10,2 10,2 10,2 10,2 10,2 10,2 10,2 10,2 10,2 10,2 10,2 10,
2 10,2 10,2 11,2 11,2 11,2 11,2 11,2 11,2 11,2 11,2 11,2 11,
2 11,2 11,2 11,2 11,2 11,2 11,2 11,2 11,2 11,2 11,2 11,2 11,
2 11,2 11,2 11,2 11,2 11,2 11,2 11,2 11,2 11,2 12,2 12,2 12,
2 12,2 12,2 12,2 12,2 12,2 12,2 12,2 12,2 12,2 12,2 12,2 12,
2 12,2 12,2 12,2 12,2 12,2 12,2 12,2 12,2 12,2 12,2 12,2 12,
2 12,2 12,2 12,2 12,2 12,2 12,2 12,2 12,2 12,2 12,2 12,2 12,
2 12,2 12,2 12,2 12,2 12,2 12,2 12,2 12,2 12,2 12,2 12,2 12,
2 12,2 12,2 12,2 12,2 12,2 12,3 13,3 13,3 14,3 14,3 14,3 15,
4 16,4 16,4 16,4 16,4 16,4 16,4 16,4 16,4 16,4 16,4 16,4 16,
4 16,4 16,4 16,4 16,4 16,4 16,4 16,4 16,4 16,4 16,4 16,4 16,
4 17,4 18,4 18,4 18,4 18,4 18,4 18,4 18,4 18,4 18,4 18,4 18,
4 18,4 18,4 18,4 18,4 18,4 18,4 18,4 18,4 18,4 18,4 18,4 18,
4 18,4 18,4 18,4 18,4 18,4 19,4 19,4 19,4 19,4 19,4 19,4 19,
4 19,4 19,4 19,4 19,4 19,4 19,4 19,4 19,4 19};

a1 = j(nat,1,0);
run ainv(rel,a1,nanim);
inva = j(nanim,nanim,0);
do i = 1 to nanim;
do j = 1 to nanim;
      run ihmssf(i,j,nanim,code);
      inva[i,j] = a1[code];
end;
end;
```

```
free a1;
a1 = inva;
free inva;

/* read-in length data for randomPC  */
yij={3.40,3.80,4.20,3.30,2.30,4.10,3.80,3.00,2.70,3.00,2.60,
3.70,3.90,5.20,4.20,5.60,3.50,4.90,4.00,2.90,6.70,5.30,4.80,
2.70,3.70,4.30,3.60,3.80,5.50,7.10,4.50,2.30,3.90,5.80,5.80,
5.40,2.70,4.50,4.30,3.50,4.70,3.90,3.50,5.00,5.20,3.00,3.00,
5.10,4.20,3.20,5.50,3.60,5.40,3.90,5.50,4.30,4.10,4.10,4.10,
5.30,6.90,4.20,4.00,6.70,4.30,4.50,6.30,2.90,5.00,4.70,4.90,
4.50,4.70,3.20,5.10,2.90,3.70,5.00,6.50,7.80,3.80,5.00,5.10,
4.60,4.50,4.50,4.90,3.90,5.50,2.90,5.00,4.30,5.30,3.50,3.50,
3.20,4.90,5.20,6.40,5.30,4.10,3.80,4.00,2.40,5.00,5.00,3.70,
4.00,4.70,5.10,5.10,4.80,2.60,3.70,5.80,4.80,6.00,4.20,3.90,
5.40,6.00,5.30,6.50,5.50,4.40,4.30,4.30,4.50,3.50,3.60,4.00,
3.00,3.20,5.40,6.60,5.20,5.40,3.00,5.00,4.60,4.70,5.80,6.20,
4.20,4.50,4.00,2.80,5.50,4.00,4.90,2.90,4.50,6.90,3.40,3.10,
2.50,5.00,4.40,5.20,2.50,4.30,2.30,3.20,4.70,2.60,3.50,3.60,
5.40,4.00,3.60,2.50,3.20,3.80,6.00,4.70,2.70,3.00,3.00,4.60,
3.60,3.70,3.80,7.70,5.80,2.90,3.60,4.10,3.50,6.20,4.50,4.30,
4.50,4.70,3.60,6.00,4.50,3.10,5.20,6.20,4.50,4.50,6.00,5.10,
2.50,4.00,4.70,4.00,4.30,3.60,3.80,5.80,6.70,3.30,3.90,4.60,
3.50,5.10,4.60,5.40,3.80,4.10,2.50,3.90,3.20,3.50,2.90,2.50,
2.90,2.70,2.70,2.30,4.50,3.90,5.60,2.90,3.20,2.50,2.20,5.20,
3.90,4.30,3.10,5.70,3.20,2.60,2.40,4.90,2.40,2.40,2.80,2.60,
3.20,3.60,4.30,3.40,2.50,3.00,2.50,2.70,2.10,3.00,3.30,4.30,
3.20,5.30,3.70,3.60,3.10,5.20,4.20,2.70,3.90,3.40,4.90,3.10,
3.00,3.70,3.40,3.40,2.70,3.10,2.80,3.00,2.70,4.70,5.80,4.00,
2.50,3.00,3.00,2.70,4.70,2.70,3.80,3.60,3.10,2.90,2.70,2.80,
4.90};

x = j(nrec,1,1);
junk = j(nrec,nadu,0);
z1 = junk||i(nrec);
w = x||z1;

free z1 x;
f = w`*yij;
yy = yij`*yij;
lslhs = w`*w;
free w yij;

run like(lslhs,f,a1,ratio,neq,nanim,nrec,nf,L1,yy,estve);

va = ratio[1]*estve;
vp = va+ estve;
h2 = va/vp;
print ratio L1 va vp estve h2;
end;
```

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