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# 論 文 内 容 要 旨

## 1. General introduction on the Mekong River and the seven-line barb

The Mekong River Basin is one of the most biologically diverse inland water systems in the world, and is the home of some 1,700 fish species. The seven-line barb, *Probarbus jullieni* Sauvage 1880, is one of the most important indigenous fish of the Mekong River and is considered as a flagship species in the content of conservation. It has been classified as endangered on the IUCN Redlist since 1996. In Thailand, the fish is found in Mae Klong River in the western part and in the Mekong River in the northeastern part of the country. However, natural populations have been extirpated from the Mae Klong River and can be expected to disappear as more impoundments are constructed in the Mekong River. At present, it is not known whether the fish that occur in various parts of the Mekong River are genetically the same or not. Therefore, it is urgently important to develop the breeding technique and examine the genetic diversity of seven-line barb, both wild and hatchery populations, in order to develop a management plan to improve the situation of the species.

## 2. Application of breeding techniques developed on Thai carp, *Barbonymous gonionotus*, to the seven-line barb

Previous studies on induced spawning of the Thai carp indicated that the gonadotropin (GtH) secretion and spawning induction in tropical cyprinids is regulated by a dual control of gondaotropin releasing hormone and dopamine. The use of gonadotropin releasing hormone analog (GnRHA) in combination with a dopamine antagonist such as domperidone (DOM) has proven to be very effective in spawning induction in the Thai carp. In the present study, this technique was tested in seven-line barb. The result showed that using Buserelin (BUS), a mammalian GnRHA, in combination with DOM alone was not as effective as using pituitary gland (PG) or using two injections of BUS+DOM and PG. Additional of gonadoplex (GDP), a mammalian GtH, could not increase the effectiveness if PG was sufficiently given. The most effective method for induced ovulation of the seven-line barb was to use two injections of 10 μg/kg BUS+10 mg/kg DOM for the first injection and 6 hrs after, 1.5-2.5 dosages of PG (Table 1). Furthermore, multiple injections of 500 IU/kg HCG to increase the oocyte diameter of female fish to ≥1.7 mm follow by two injections of BUS in combination

with DOM was found to be the most potent technique for inducing spawning of captive seven-line barb (Table 2).

**3. Identification and characterization of microsatellite DNA markers developed in the seven-line barb**

Microsatellite DNA markers for seven-line barb were developed from the wild caught samples using $(GT)_{15}$ probe (Table 3). The number of alleles per locus ranged from 7 to 16. The expected heterozygosities ranged from 0.47 to 0.91. Also, these primers were successfully amplified in the two closely related species, *P. labeamajor* and *P. labeaminor*. These markers have proven to be very useful for the population genetic structure study in this species and other related cyprinids.

**4. Assessment of genetic diversity of the wild seven-line barb in the Mekong River based on microsatellite and mitochondrial DNA markers**

Genetic diversity of seven-line barb was investigated using 6 msDNA markers and sequencing analysis of mitochondrial DNA control region (mtDNA). Both msDNA and mtDNA showed concordant results in demonstrating similar genetic diversities and population structuring of seven-line barb (Table 4). Furthermore, genetic diversity of seven-line barb was quite high compared with other endangered fish species reported to date. Population sub-structuring of seven-line barb was also observed (Table 5 and Figure 1).which was no evidence for isolation by distance ($r=0.712$, $p=0.113$) and no apparent physical barriers. The results suggested that the number of populations of seven-line barb may be determined by the number of geographic setting within which their life cycle can be completed. For management point of view, it is proposed that all populations should be directed toward preserving the genetic integrity of each group.

**5. Evaluation of genetic diversity in hatchery populations of seven-line barb based on microsatellite and mitochondrial DNA markers**

Genetic diversity within and between hatchery populations of seven-line barb was compared with those of wild populations using 5 microsatellite DNA markers

(msDNA) and sequencing analysis of the mitochondrial DNA control region (mtDNA). Both msDNA and mtDNA markers showed comparable results in demonstrating similar genetic diversity of seven-line barb (Table 6). Marked reductions of genetic variability in the hatchery populations compared with the wild populations were observed in term of both msDNA number of alleles and mtDNA haplotypes. $F_{ST}$ and $\Phi_{ST}$ values also suggested that the magnitude of the genetics divergence within and among wild and hatchery populations of seven-line barb was significant (Table 7 and Figure 2). The lower genetic variability observed in the hatchery populations may cause by bottleneck effect due to the limited number of effective parents when each population was founded. Enhancing genetic variability and eliminating the accumulated effect of inbreeding of hatchery populations by means of increasing effective population size, preventing inbreeding and using selective breeding based on the principle of minimal kinship broodstock management is recommended to minimize genetic impacts of restocking program.

**6. Simulation for preserving genetic diversity of hatchery populations of the seven-line barb by minimal kinship selective breeding**

The effectiveness of the minimal kinship selective breeding and random breeding on preservation of genetic diversity of the hatchery populations of seven-line barb was compared. The results indicated that the calculated by *Psa* values (*MKp*), based on 5 msDNA markers, yielded a better result in retaining the loss of $H_E$ and $A_E$ than the random selection (Table 8). For $H_E$, the differences of both approaches were prominent when the number of parent used in each generation was more than 10 pairs. Further, the loss of $H_E$ significantly decreased when the number of parent increased (Kruskal Wallis test, $p<0.05$). The *MKp* approach could increase about 6.4% of $H_E$ within 30 generations when at least 100 parents were used and 500 offspring were kept in each generation (Figure 3). Moreover, the *MKp* selective breeding showed a better result in retaining the loss of $A_E$ only when the number of parent was at 100 pairs. *MKp* selective breeding approach could conserve about 80% of $A_E$ for 30 generations when at least 500 offspring was kept (O=500) and 100 parents (P=100) were used as breeders in each generation (Figure 4). Furthermore, if the number of parent was set at 100 individuals, inbreeding will not occur in the *MKp* selective

breeding approach (Figure 5). For preserving genetic diversity of seven-line barb, it is recommended to use minimal kinship selective breeding approach which uses 100 parents and keep 500 offspring in each generation.

## 7. Conclusion and recommendations on broodstock management of seven-line barb based on DNA markers and computer simulation

From the results, the conclusion and recommendation on broodstock management of seven-line barb are summarized in Figure 6 as follow:

1. Because the seven-line barb in the Mekong River exhibits the genetics divergence according to their spawning grounds, each population could be considered as "Management Units". All population should be directed towards preserving the genetic integrity of each group.

2. Since the genetic diversity of hatchery populations was different from the wild populations, it should not be used for restocking. Captive broodstock used for restocking program should be founded by collecting fish from the wild populations, and the offspring should be released back into natural habitat from which the founders were derived. These broodstock could also be used for development of aquaculture.

3. Breeding of the seven-line barb should be done by using minimal kinship selective breeding approach in order to retain genetic diversity of the captive stock.

4. Induced spawning of seven-line barb should be done using multiple injections with hCG to increase the oocyte diameter to $\geq$ 1.7 mm follow by 2 injections of BUS and DOM.

5. Genetic variability of seed fish should be examined before restocking and/or aquaculture used.

6. Genetic diversity of both wild and hatchery populations should be monitored regularly in order to assess the success of its management plan.

**Table 1.** Effect of various types of hormone on induced spawning of captive *P. jullieni*

| Group | Type and Concentration of Hormone | | Proportion of fish spawned | Average wt. of egg stripped per fish (g) | Fertilization rate (%) | Hatching rate (%) |
|---|---|---|---|---|---|---|
| | First Injection | Second Injection | | | | |
| 1 | PG 0.5 | PG 1.5 | 5:5 | 138.40±48.20 | 20.10±8.45 | 52.00±1.25 |
| 2 | BUS 10 + DOM 5 | BUS 10 + DOM | 0:5 | - | - | - |
| 3 | BUS 30 + DOM 15 | - | - | - | - | - |
| 4 | BUS 10 + DOM 5 | PG 1.5 | 5:5 | 30.00±0.00 | 67.96±10.75 | 64.00±3.75 |
| 5 | BUS 10 + DOM 5 | PG 1.5 + GNP 30 | 5:5 | 290.20±56.80 | 89.88±15.25 | 84.00±1.75 |
| 6 | BUS 10 + DOM 5 | PG 2.5 | 5:5 | 280.60±27.50 | 90.75±8.80 | 92.00±3.75 |

**Table 2.** Effects of multiple injections of hCG on inducing final maturation and ovulation in seven-line barb

| Priming injection[1] | avg. body wt (kg) | Resolving injection[2] | | Proportion of fish spawned[3] | Latency period[4] |
|---|---|---|---|---|---|
| | | First injection | Second injection | | |
| None | 4.22±0.35 | PG 0.5 | PG 2.0 | 1:5 | 4±0.5 |
| None | 4.16±0.63 | BUS 5+DOM 5 | BUS 20+DOM 10 | 1:5 | 6±1.2 |
| None | 4.40±0.64 | BUS 30+DOM 10 | - | 0:5 | - |
| 3-5 | 4.60±0.94 | hCG 500 IU | hCG 2500 IU | 2:5 | 4±0.2 |
| 3-5 | 4.54±0.61 | hCG 1000 IU | hCG 2500 IU | 3:5 | 4±0.3 |
| 3-5 | 4.56±0.87 | hCG 3000 IU | - | 2:5 | 3±1.2 |
| 3-5 | 4.46±0.55 | PG 0.5 | PG 2.0 | 2:5 | 4±0.7 |
| 3-5 | 4.92±0.67 | BUS 5+DOM 5 | BUS 20+DOM 10 | 5:5 | 6±1.5 |
| 3-5 | 4.72±0.70 | BUS 30+DOM 10 | - | 4:5 | 5±1.8 |

PG =Pituitary gland (dose), BUS= Buserelin (μg/kg), DOM= domperidone (mg/kg), hCG=human Chorionic gonadotropic

1. Number of hCG priming injection (range values), one injection per day at 10.00 am at a dose of 500 IU/kg
2. First and second injection is 6 hr interval
3. Number of ovulated females:number of treated females
4. Delay (hrs) between second injection and ovulation

**Table 3.** Designed microsatelliet DNA markers, observed number of alleles per locus, observed ($H_O$) and expected ($H_E$) heterozygosities in seven-line barb and DDBJ accession number

| Locus | No. of observed Alleles | $H_O$ | $H_E$ | DDBJ Accession no. |
|---|---|---|---|---|
| *Proju 1* | 14 | 0.90 | 0.68 | AB167402 |
| *Proju 3* | 7 | 0.72 | 0.68 | AB167403 |
| *Proju 6* | 8 | 0.80 | 0.91 | AB167404 |
| *Proju 8* | 16 | 0.92 | 0.84 | AB167405 |
| *Proju 9* | 9 | 0.83 | 0.86 | AB167406 |
| *Proju 12* | 13 | 0.92 | 0.47 | AB167407 |

**Table 4:** Summary of genetic variation based on msDNA and msDNA data in 4 seven-line barb populations in the Mekong River. N=number of sample, $H_O$=observed heterozygosity, $H_E$=expected heterozygosity

| Population | N | Allele richness | $H_O$ | $H_E$ | Haplotype diversity | Nucleotide diversity |
|---|---|---|---|---|---|---|
| LOEI | 40 | 12.42 | 0.72 | 0.86 | 0.97 | 0.018 |
| NK | 44 | 12.35 | 0.68 | 0.85 | 0.85 | 0.03 |
| MK | 46 | 12.76 | 0.77 | 0.86 | 0.91 | 0.013 |
| UB | 58 | 11.83 | 0.68 | 0.83 | 0.97 | 0.023 |

LOEI= Loei population, NK= Nongkhai population, MK= Mukdaharn population
UB= Ubolratchathani population

**Table 5:** Estimated pairwise $F_{ST}$ (below diagonal) and $R_{ST}$ (above diagonal) values based on msDNA among 4 wild populations of seven-line barb

| | LOEI | NK | MUK | UB |
|---|---|---|---|---|
| LOEI | - | 0.1088* | 0.07826* | 0.02528* |
| NK | 0.0125* | - | -0.0195 | 0.03638* |
| MUK | 0.0198* | 0.0078 | - | 0.02483* |
| UB | 0.0021 | 0.0144* | 0.0064 | - |

P=Significant level based on random allelic permutation testing.
Overall $F_{ST}$=0,01604, p=0.0000; overall $R_{ST}$=0.08108, p=0.0000

**Table 6.** Summary of genetic variation based on msDNA and msDNA data in hatchery and wild seven-line barb populations. N=number of sample, $H_O$=observed heterozygosity, $H_E$=expected heterozygosity

| Population | N | Allelic richness | $H_O$ | $H_E$ | Haplotype diversity | Nucleotide diversity |
|---|---|---|---|---|---|---|
| NKH | 56 | 8.56 | 0.72 | 0.78 | 0.5 | 0.002 |
| NPH | 25 | 8.6 | 0.74 | 0.82 | 0.71 | 0.004 |
| MKH | 60 | 6.56 | 0.52 | 0.62 | 0.32 | 0.002 |
| SH | 46 | 5.41 | 0.78 | 0.69 | 0.32 | 0.003 |
| LOW | 40 | 11.67 | 0.74 | 0.84 | 0.97 | 0.018 |
| NKW | 44 | 10.79 | 0.73 | 0.83 | 0.85 | 0.032 |
| MKW | 46 | 12.01 | 0.81 | 0.84 | 0.91 | 0.013 |
| UBW | 58 | 10.83 | 0.72 | 0.82 | 0.97 | 0.024 |

NKH= Nongkhai Hatchery, NPH= Nakornphanom Hatchery, MKH= Mukdaharn Hatchery, SH= Surin Hatchery, LOW= Loei wild population, NKW= Nongkhai wild population, MKW= Mukdaharn wild population, UBW= Ubolratchathani wild population

**Table 7:** Estimates of $F_{ST}$ and $\Phi_{ST}$ values based on msDNA and mtDNA sequences

| | msDNA | | mtDNA | |
|---|---|---|---|---|
| | $F_{ST}$ | P | $\Phi_{ST}$ | P |
| Global (no subdivision) | 0.0801 | 0.0000 | 0.0890 | 0.0000 |
| Among hatchery populations | 0.0964 | 0.0000 | 0.1288 | 0.0000 |
| Among wild populations | 0.0160 | 0.0000 | 0.0477 | 0.0003 |
| Hatchery vs wild population | 0.0977 | 0.0000 | 0.0617 | 0.0000 |
| NKH-NKW | 0.0602 | 0.0000 | 0.2372 | 0.0000 |
| MKH-MKW | 0.1437 | 0.0000 | 0.0352 | 0.0029 |

P=Significant level based on random allelic permutation testing.

**Table 8.** Simulation of genetic variability as determined by expected heterozygosity ($H_E$) and average number of alleles over loci ($A_E$) of the 4 hatchery populations of the seven-line barb after 30 generations ($F_{30}$) under minimal kinship selective breeding (MK) and random selective breeding (Random). $G_0$=founder generation.

| Hatchery | P | O | Heterozygosity ($H_E$) | | | | |
|---|---|---|---|---|---|---|---|
| | | | $G_0$ | MK ($F_{30}$) | % loss | Random ($F_{30}$) | % loss |
| NKH | 20 | 500 | 0.78±0.00 | 0.57±0.03 | 27.3 | 0.67±0.05 | 14.4 |
| n=56 | 50 | 500 | 0.78±0.00 | 0.73±0.05 | 7.18 | 0.59±0.06 | 24.7 |
| | 100 | 500 | 0.78±0.00 | 0.84±0.00 | *-7.4* | 0.67±0.04 | 14.0 |
| NPH | 20 | 500 | 0.82±0.00 | 0.58±0.03 | 29.5 | 0.69±0.37 | 15.0 |
| n=25 | 50 | 500 | 0.82±0.00 | 0.71±0.04 | 13.5 | 0.60±0.68 | 26.4 |
| | 100 | 500 | 0.82±0.00 | 0.83±0.02 | *-1.8* | 0.69±0.42 | 15.6 |
| SRH | 20 | 500 | 0.69±0.00 | 0.55±0.03 | 21.1 | 0.60±0.27 | 12.9 |
| n=46 | 50 | 500 | 0.69±0.00 | 0.63±0.03 | 8.89 | 0.51±0.38 | 26.7 |
| | 100 | 500 | 0.69±0.00 | 0.67±0.03 | 3.38 | 0.59±0.35 | 14.3 |
| MKH | 20 | 500 | 0.62±0.00 | 0.59±0.04 | 5.73 | 0.54±0.05 | 13.9 |
| n=61 | 50 | 500 | 0.62±0.00 | 0.65±0.03 | *-4.1* | 0.45±0.07 | 27.1 |
| | 100 | 500 | 0.62±0.00 | 0.75±0.05 | 19.7 | 0.53±0.05 | 14.4 |

| Hatchery | P | O | Average number of allele per loci ($A_E$) | | | | |
|---|---|---|---|---|---|---|---|
| | | | $G_0$ | MK ($F_{30}$) | % loss | Random ($F_{30}$) | % loss |
| NKH | 20 | 500 | 10.0±0.00 | 2.80±0.36 | 72.0 | 5.53±0.46 | 44.7 |
| n=56 | 50 | 500 | 10.0±0.00 | 5.61±0.82 | 43.9 | 3.98±0.54 | 60.2 |
| | 100 | 500 | 10.0±0.00 | 7.97±0.32 | 20.3 | 5.53±0.58 | 44.7 |
| NPH | 20 | 500 | 8.6±0.00 | 2.93±0.37 | 66.0 | 5.83±0.38 | 32.2 |
| n=25 | 50 | 500 | 8.6±0.00 | 5.21±0.68 | 39.4 | 4.16±0.45 | 51.6 |
| | 100 | 500 | 8.6±0.00 | 7.57±0.42 | 12.0 | 5.68±0.39 | 34.0 |
| SRH | 20 | 500 | 6.0±0.00 | 2.52±0.27 | 58 | 4.18±0.36 | 30.3 |
| n=46 | 50 | 500 | 6.0±0.00 | 3.74±0.38 | 37.6 | 3.33±0.35 | 44.5 |
| | 100 | 500 | 6.0±0.00 | 4.67±0.35 | 21.9 | 4.15±0.30 | 30.9 |
| MKH | 20 | 500 | 8.6±0.00 | 3.33±0.40 | 61.3 | 4.31±2.31 | 49.9 |
| n=61 | 50 | 500 | 8.6±0.00 | 4.15±0.46 | 51.7 | 3.17±2.07 | 63.1 |
| | 100 | 500 | 8.6±0.00 | 6.32±0.61 | 26.6 | 4.23±2.01 | 50.8 |

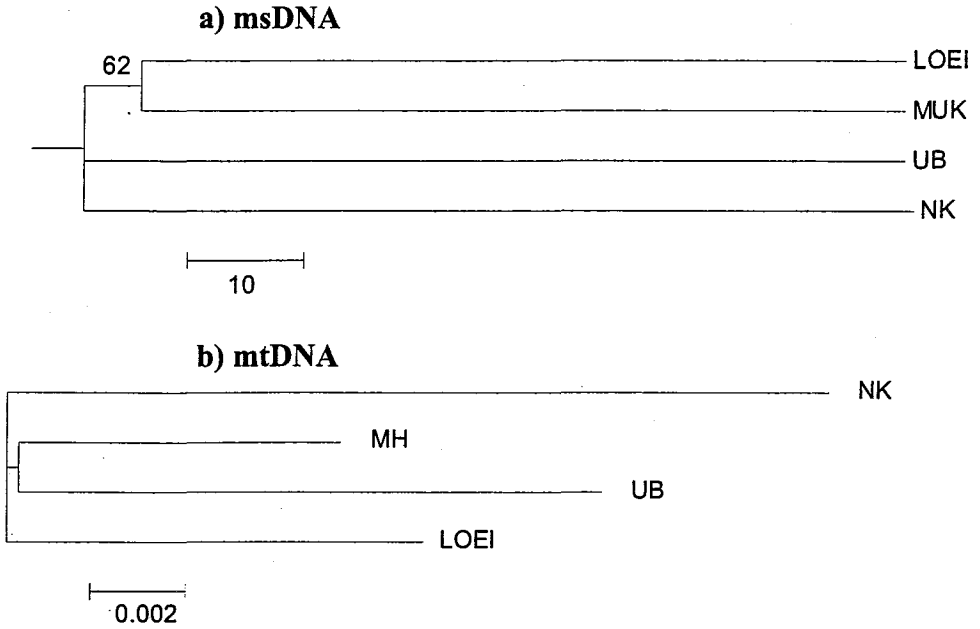


**Figure 1.** UPGMA tress from genetic distances for 4 sample regions using (a) Average Square Distance from 6 msDNA loci and (b) Tamura-Nei's model of sequence evolution from mtDNA

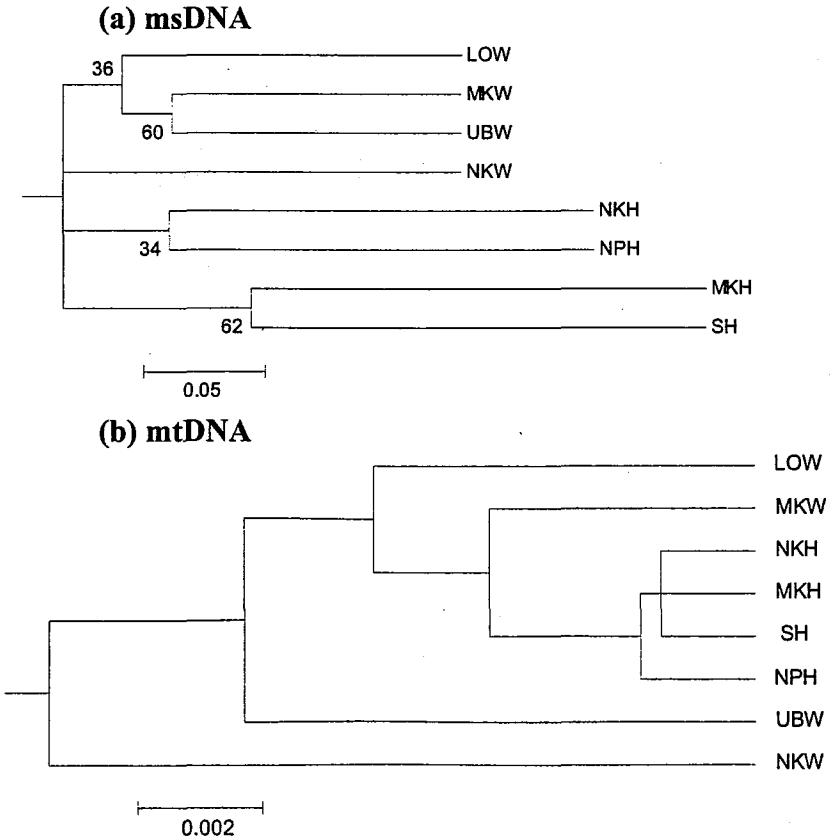


**Figure 2.** UPGMA tress from genetic distances for 8 populations of wild and hatchery using (a) Cavalli-Sforza chord distances from 5 msDNA loci and (b) Tamura-Nei's model of sequence evolution from mtDNA

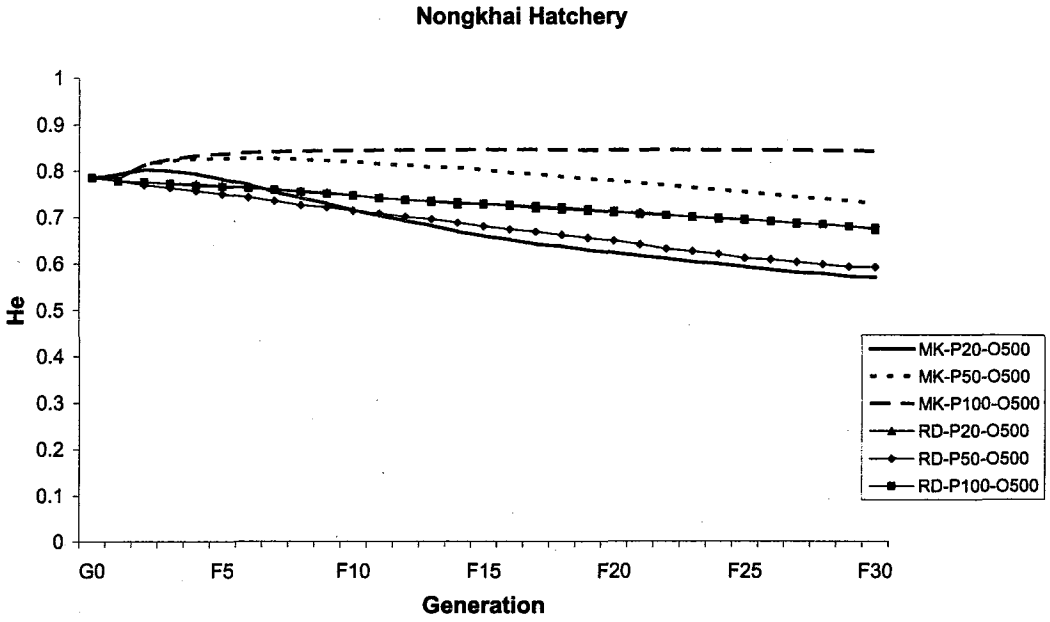


**Figure 3.** Average changes in heterozygosity ($H_E$) in Nongkhai hatchery from 50 repeated simulations for 30 generations under minimal kinship selective breeding (MK) and random selective breeding (RD) using 20, 50 and 100 individuals of parents (P) and 500 offspring (O) per generation

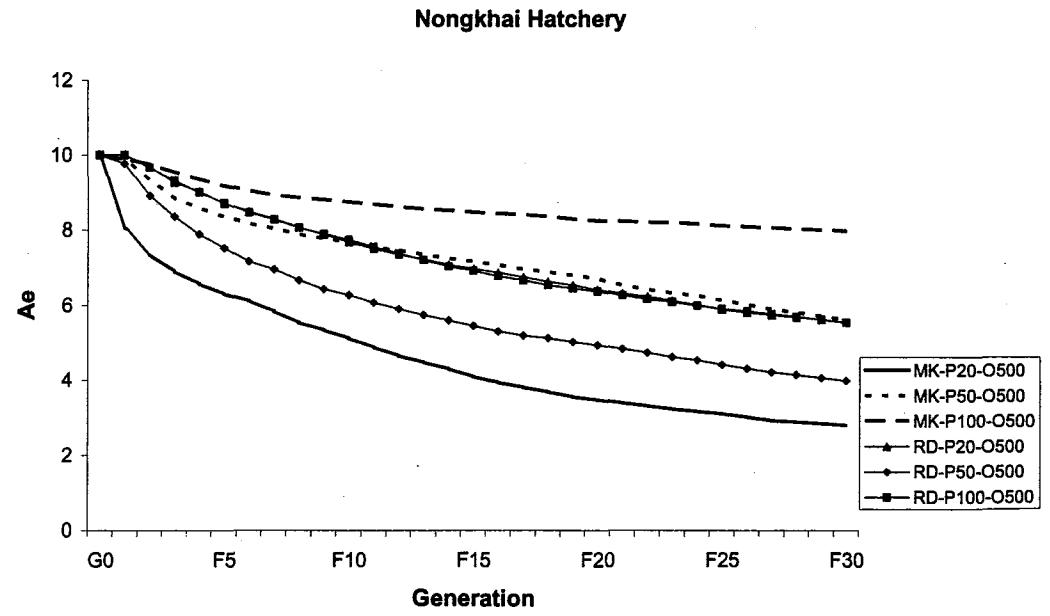


**Figure 4:** Average changes in average number of alleles ($A_E$) in Nongkhai hatchery from 50 repeated simulations for 30 generations under minimal kinship selective breeding (MK) and random selective breeding (RD) using 20, 50 and 100 individuals of parents (P) and 500 offspring (O) per generation

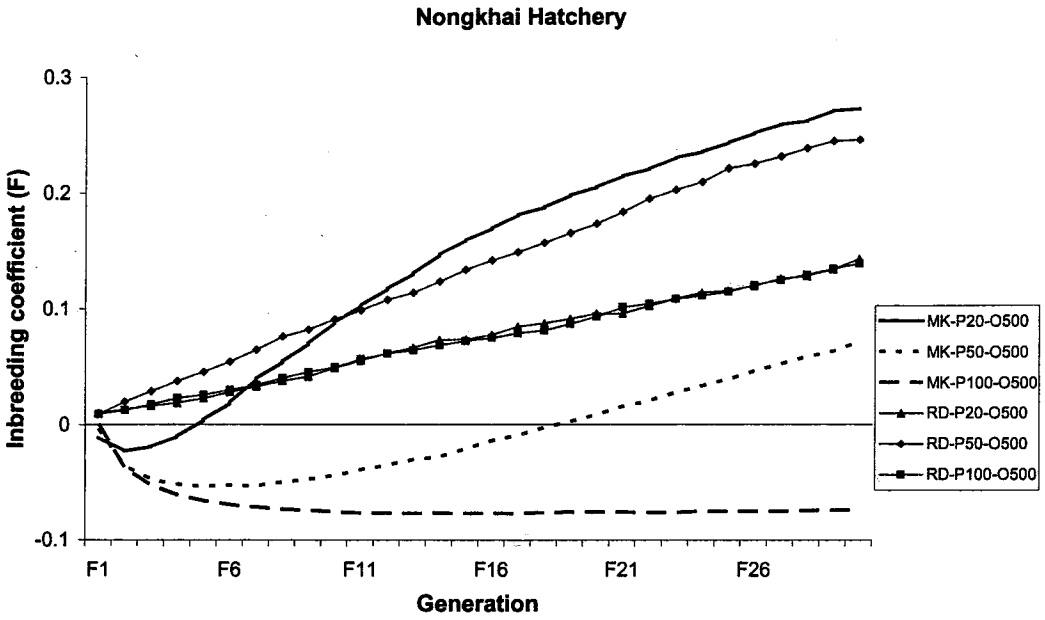


**Figure 5.** Simulation of inbreeding coefficient (F) in Nongkhai hatchery for 30 generations under minimal kinship selective breeding (MK) and random selective breeding (RD) using 20, 50 and 100 individuals of parents (P) and 500 offspring (O) per generation

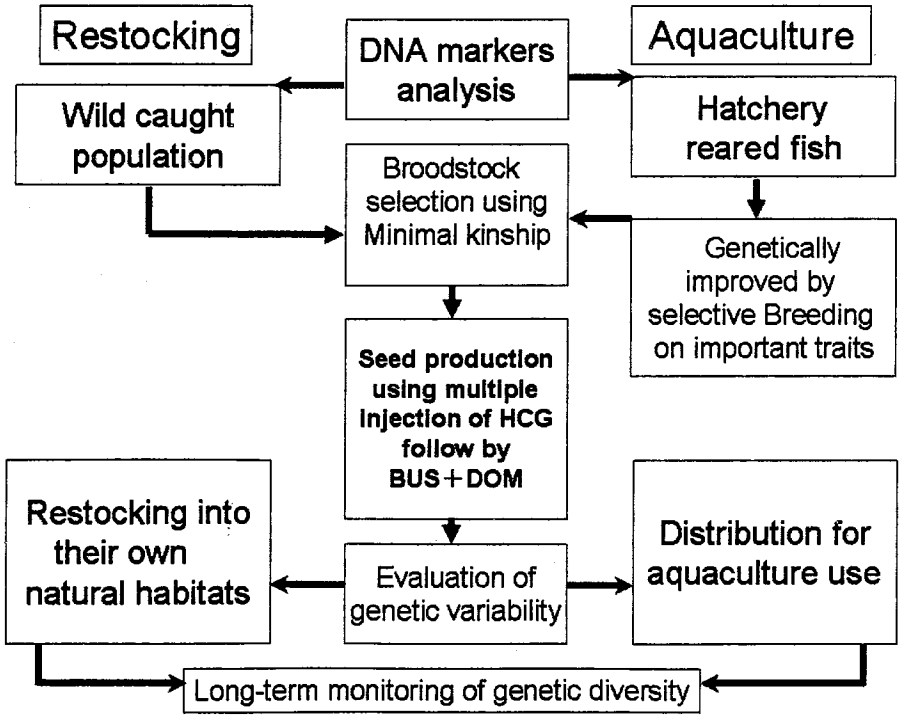


**Figure 6.** Diagram of the proposed broodstock management to minimize the loss of genetic variation in the seven-line barb for restocking and aquaculture

# 論文審査結果要旨

メコン川は東南アジア最大の河川で，その水資源および生物多様性を包含する生態系の保全は今後の流域国の社会発展を左右する重要課題となっている。コイ目の大型種である Seven-line barb（*Probarbus jullieni*）は，メコン川流域の食資源の1つとして重視されてきたが，乱獲や河川環境の変化により資源量が著しく低下し，1996年にはIUCN（国際自然保護連合）によって絶滅危惧種に指定されている。本研究は，本種が種苗生産とその放流などの増殖事業の対象となっている現状に鑑み，本種の絶滅リスクを査定・評価し，適正な遺伝的管理手法の創出を目指したものである。

最初に，本種の人工受精のための排卵誘導条件に関わる実験を，近縁の小型種の *Barbonimus gonionotus* を用いて実施し，血清ゴナドトロピン分泌と排卵の誘導のために必要な GnRHA（Gonadotropin releasing hormone）と DOM（Domperidon as a dopamine antagonist）を組み合わせる最適条件を解明した。次に，標的種の Seven-line-barb に両ホルモンを投与し，安定的に採卵出来る条件を確認した。

次に，本種の集団遺伝学的分析に必要な高感度マーカーであるマイクロサテライト DNA マーカーの開発を試み，多数のプライマーセットの中から多型性を備える6プライマーセットの作製に成功し，それらのマーカー座の遺伝的特性評価を行っている。メコン川の4地点から採集した野生集団の標本群についてマイクロサテライト DNA マーカーおよびミトコンドリア DNA マーカーを検出して，集団間の遺伝的分化が統計的に有意であることを確認し，本川内に複数の相対的に独立の集団からなる遺伝的構造の存在を推定した。また，各地の水産試験場に保持されている採卵用親魚集団を採集し，同様の集団分析を実施したところ野生集団間より大きい遺伝的分化を認めている。

多様性の低下の防止手法として，遠縁の個体を選び交配を行う，Minimal Kinship 法（MK 法）が有効と考え，継代シミュレーションを30世代にわたって行い，遺伝的多様性レベル維持に関する効果を評価・検討し，本シミュレーション法が遺伝的多様性維持のための情報を得る上で効果的であることを解明した。今後，種苗生産の現場において Minimal Kinship 選択交配を実施する際には，継代シミュレーションに基づくリスク予測を実施し，リスク防止条件を解明した後に，種苗生産とその放流を実施すべきとする管理マニュアルを提案した。

以上のように，本研究はメコン川における現存の Seven-line barb 集団の多様性を DNA マーカーによって評価し，これらのデーターを用いたシミュレーションにより，野生集団の遺伝的保全に配慮した Seven-line barb の放流事業のあり方について価値ある提言を行っている。よって，審査委員一同は本論文の著者を博士（農学）の学位を授与するに値するものと判定した。