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Intraspecific Polymorphism in *Mystus nemurus* (C&V) Detected by RAPD-PCR Fingerprinting

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ABSTRAK

Peringkat pembezaan sub-populasi genetik di kalangan sampel ikan baung, Mystus nemurus *(C&V) yang diperoleh daripada sebahagian kawasan, serta genetik populasi liar dan diternak dibandingkan. Aspek variasi genetik ikan yang dikumpul daripada lapan populasi di seluruh Thailand dan stok hatcheri ditentukan di peringkat molekul (DNA) dengan menggunakan teknik cap jari RAPD-PCR. Lima primer OPA-11, OPA-14, OPA-18, OPA-19 dan OPA-20 dipilih untuk mengamplifikasikan DNA. Ini menghasilkan 28 lokus polimorfik dalam 9 populasi yang dikaji. Jarak genetik (D) yang terbesar didapati antara populasi Chainat dan Suratthani dengan nilai 0.289, manakala jarak genetik terkecil didapati di antara pasangan populasi Songkhla dan stok hatcheri dengan nilai 0.087. Dendrogram menggambarkan perhubungan genetik di kalangan populasi M.* nemurus *yang digolongkan kepada empat kelompok mengikut kawasan asalnya.*

ABSTRACT

Yellow catfish, Mystus nemurus *(C&V), is becoming one of the major freshwater species farmed by aquaculturists in Southeast Asia. It was of interest to examine levels of genetic sub-population differentiation among samples of this species obtained from parts of its range, as well as to compare the genetics of wild and hatchery-bred fish. The genetic aspects of variation in the fish, which were collected from eight wild populations throughout Thailand and a hatchery stock, were determined at molecular (DNA) level using the technique of RAPD-PCR fingerprinting. Five arbitrary primers namely OPA-11, OPA-14, OPA-18, OPA-19 and OPA-20 were chosen to amplify products, which showed 28 polymorphic loci in 9 populations. The highest genetic distance (D) was found between Chainat and Suratthani populations with the value of 0.289, while the lowest was found in Songkhla population and hatchery stock with the value of 0.087. The dendrogram depicts the genetic relationship among populations of* M. nemurus, *which are grouped into four clusters according to their regional areas.*

INTRODUCTION

Genetic markers have been widely used for estimating genetic variation in breeding programs for many organisms. There are many methods of revealing genetic differentiation such as protein electrophoresis, DNA fingerprinting and others. Although protein electrophoresis has provided a wealth of genetic data to date, it has certain limitations. The resolution of protein electrophoresis is always inadequate for detecting differences between populations or individuals (Grant and Utter 1980). Thus, the potential amount of genetic variation detectable by DNA methods vastly exceeds the amount detectable by protein methods because DNA sequences are assayed more directly (Park and Moran 1994). There are various techniques used for the analysis of DNA level variation such as Random Amplified

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Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), Restriction Fragment Length Polymorphism (RFLP) and microsatellites. In 1990, a method of revealing DNA-based polymorphisms named Random Amplified Polymorphic DNA (RAPD) fingerprinting, which involves PCR amplification of genomic DNA using a single primer of arbitrary nucleotide sequence, was reported (Welsh and McClelland 1990; Williams *et al.* 1990). Numerous studies on genetic polymorphisms in various organisms using RAPD fingerprinting have now been documented. In botany, RAPD markers were used in strawberry and many plant species (Brown *et al.* 1993; Davis and Yu 1997; Schierenbeck *et al.* 1997). RAPD have been used in fishes for species identification in tilapia (Bardakci and Skibinski 1994), guppy (Foo *et al.* 1995), sea bass (Caccone *et al.* 1997), *Anguilla sp.* (Takagi and Taniguchi 1995) and *Liobagrus reini* (Na-nakorn *et al.* 1996). Several studies on RAPD markers in the yellow catfish, *Mystus nemurus*, have also been reported (Chong 1998; Foo 1998).

In this study, the genetic variations in yellow catfish, *Mystus nemurus* (*Fig. 1*), collected from eight wild populations throughout Thailand and a hatchery stock were determined at the molecular (DNA) level using the RAPD-PCR fingerprinting technique. The results provided, for the first time, data at the DNA level on the genetic structure of natural and hatchery populations of this species in Thailand. The information obtained will be useful for fish population identification, formulation of programs for breeding, and improvement for culture activities, and other aquacultural development programs in the future.

MATERIALS AND METHODS

Sample Collection

Thirty to fifty specimens of *M. nemurus* ranging from 10.5 to 41.0 cm in length and 7.0 to 781.3 g in weight were collected from 8 different locations in Thailand (*Fig. 2*). A hatchery stock was also obtained from the hatchery operated by the Suratthani Inland Fisheries Development Center (SIFDC), in southern Thailand. Live fish were transported from the local areas to the nearest fishery station for tissue collection. Flank muscle tissues of each sample were collected and then stored at – 80°C before being transported to the Genetics Laboratory, Department of Biology, Faculty of Science and Environmental Studies, Universiti Putra Malaysia using dry ice where laboratory experiments were conducted.

RAPD Procedure and Analysis

Extraction of genomic DNA: The flank muscle tissues from each individual were pulverized after thawing. The genomic DNA was extracted using the WIZARD™ a DNA purification kit, which was supplied by Promega Corporation. Six hundred microliters of Nuclei Lysis Solution were added to a 1.5 ml centrifuge tube and chilled on ice. Next, 10-20 mg ground tissue were transferred into the solution and gently mixed. The mixture was then incubated in a water bath at 65°C for 30 minutes. Three microlitres of RNaseA solution were added to the lysate, and then mixed by inverting the tube for 25 times before incubating in water bath at 37°C for 30 minutes. The mixture was then allowed to cool to room temperature for 5 minutes. Two hundred microlitres of Protein Precipitation Solution were added to RNaseA

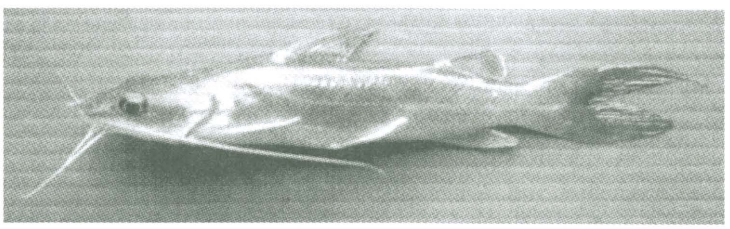

Fig. 1: Yellow catfish, Mystus nemurus (C&V)

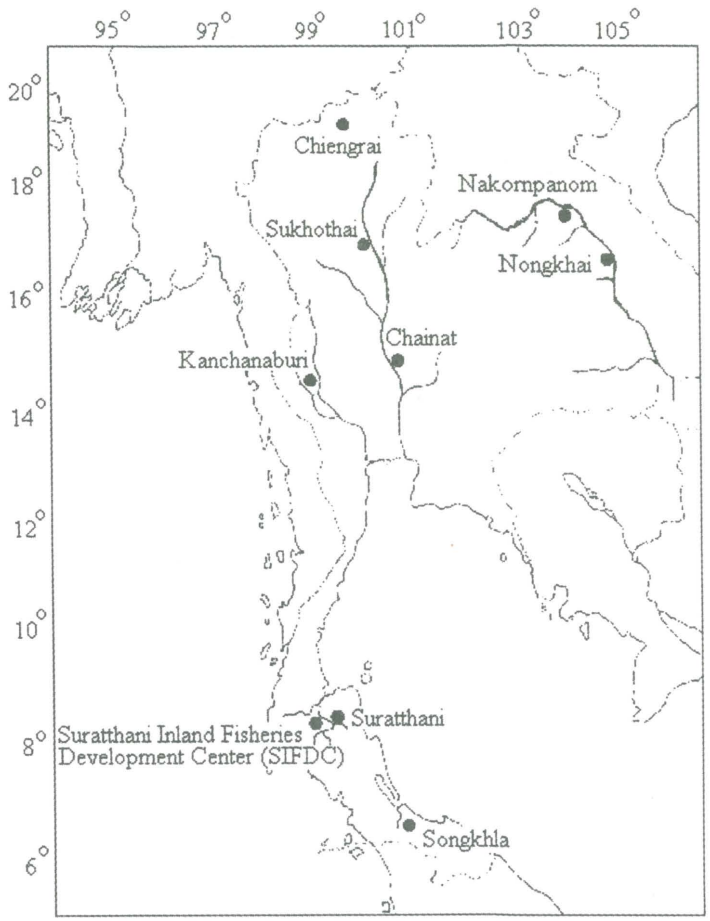

Fig. 2: Sampling locations of Mystus nemurus in Thailand

treated cells and vortexed vigorously at high speed for 20 seconds. The mixture was centrifuged for 3 minutes at 13,000-16,000 X g. After that, the supernatant containing the DNA was carefully removed (leave the residual liquid in the tube to avoid contaminating the DNA with the precipitated protein) and transferred to a clean 1.5 ml centrifuge tube containing 600 ml of room temperature isopropanol, then the solution was gently mixed by inversion until the white thread-like strands of DNA formed a visible mass. The solution then was centrifuged at 13,000-16,000X g for 1 minute. The DNA was visible as a small white pellet. The supernatant was then carefully decanted. The pelleted DNA obtained was then washed twice with 600 μl of 70% ethanol, and the tube inverted several times to clean up the DNA. The tube was then centrifuged at 13,000-16,000 X g for 1 minute. The solution was carefully poured off. The DNA pellet was dried at air temperature for 15 minutes. DNA Rehydration Solution (100 μl) was added to the DNA pellet and the solution was incubated overnight at room temperature. The DNA sample was then stored at 2-8°C until used. The extracted DNA was quantified by comparing its intensity to the intensities of a range of diluted DNA using 0.8 % horizontal agarose gel electrophoresis in 1X TBE buffer (0.045 M Tris-borate and 1 mM EDTA, pH 8.0) at 70 V for 1 hour.

Polymerase Chain Reaction: A total of 20 arbitrary primers from Kit A (Operon Technologies, USA) were screened. Only 5 arbitrary primers, which seemed to work well were selected for the study. The DNA amplification was performed following the procedure recommended by William *et al.* (1990) with slight modifications. The reactions were performed in a volume of 25 μl containing 10 mM Tris HCl; 50 mM KCl; 2.5 mM $MgCl_2$; 0.2 mM each of dATP, dGTP, dCTP and dTTP (Promega); 5 pmol of a single primer; 50 ng of genomic DNA;

TABLE 1
Number of fish, total length (cm) and body weight (g) of yellow catfish, *M. nemurus*, from different localities in Thailand

Sampling site in Thailand	Number of fish	Total length (cm) ± S.D.			body weight (g) ± S.D.		
	Min.	Max.	Average	Min.	Max.	Average	
North							
1. Chiengrai	30	13.2	20.7	16.00±1.68	15.0	55.0	25.20±8.39
2. Sukhothai	30	21.1	26.6	24.10±1.39	60.0	135.0	93.10±19.13
Central							
3. Kanchanaburi	30	22.8	39.0	27.70±3.36	150.0	365.0	230.20±60.72
4. Chainat	30	15.5	24.3	19.50±2.01	35.0	115.0	68.10±19.12
North-east							
5. Nongkhai	47	12.2	35.5	21.00±4.85	13.0	306.0	75.90±52.25
6. Nakornpanom	46	10.5	25.5	16.20±4.13	7.0	115.0	37.40±28.96
South							
7. Suratthani	50	20.2	41.0	29.40±4.74	68.9	781.3	214.20±132.92
8. Songkhla	50	21.1	39.5	27.20±3.52	58.6	444.5	140.40±64.69
Hatchery stock							
9. Suratthani Inland Fisheries Development Center (SIFDC)	50	20.5	31.5	26.60±2.57	66.5	250.7	144.30±37.31

and 2 unit of *Taq* DNA polymerase (Promega USA). Amplification was done in a Perkin-Elmer-Cetus model 2400 thermocycler. The amplification was performed as follows: predenaturation at 92°C for 2 min; followed by 40 cycles at 94°C for 30 sec, 40°C for 30 sec, 72°C for 1 min, and a final extension step at 72°C for 5 min (Chong 1998). The PCR products were kept at 4°C until subjected to electrophoretic analysis.

Agarose gel electrophoresis: The PCR products were separated by electrophoresis on a 1.8 % horizontal agarose gel. For each gel, 5 µl of a 100 bp DNA ladder (Promega USA) was used as a molecular weight standard. The gels were electrophoresed in TBE buffer at 90 V for 2 to 4 hours depending on the sizes of the amplified fragments for each primer. After electrophoresis, the gels were soaked in 1 µg/ml ethidium bromide in 1X TBE buffer for 10 to 15 minutes. After that, the gels were twice rinsed in distilled water and photographed on an ultraviolet transilluminator using polaroid film before data interpretation.

Data interpretation and analysis: The data scoring was based on observed presence or absence of bands. The data from individual samples in each population were used to calculate Nei and Li's (1979) similarity index and to produce an UPGMA dendrogram of genetic relationships based on genetic distances. These analyses were facilitated by using the "NTSYS-pc" (Version 1.8) computer program (Rohlf 1993).

RESULTS

As a preliminary step, a total of 20 primers, OPA-1–20, were screened for PCR amplification. Two primers (10 %), OPA-8 and OPA-12 failed to amplify products of sufficient quality for analysis. Only 18 RAPD primers (90 %) yielded good amplification products. Of these primers, OPA-11, OPA-14, OPA-18, OPA-19 and OPA-20 (Table 2) yielded PCR products which produced clear banding patterns and were selected for the detection of genetic variation in eight wild populations and a hatchery stock of *M. nemurus*.

The five selected primers produced a total of 46 scorable bands ranging in sizes from 380 to 1,550 bp. Each primer generated between 4-16 scorable bands. The complexity of the banding patterns varied among primers. Primer OPA-18

TABLE 2
Primer codes and sequences used to study polymorphism of *Mystus nemurus* (C& V)

Primer names	Primer sequences (5' to 3')
OPA-11	CAATCGCCGT
OPA-14	TCTGTGCTGG
OPA-18	AGGTGACCGT
OPA-19	CAAACGTCGG
OPA-20	GTTGCGATCC

TABLE 3
Total number of amplified bands for each primer with size range of amplified fragments (bp) in eight wild populations and a hatchery stock of *M. nemurus* revealed by RAPD fingerprinting

Primer No.	Size-range	No. of amplified bands	No. of monomorphic bands*	No. of polymorphic bands
OPA-11	750 – 1060	6	2 (33.33 %)	4 (66.67 %)
OPA-14	460 – 1550	12	6 (50.00 %)	6 (50.00 %)
OPA-18	380 – 1240	16	6 (37.50 %)	10 (62.50 %)
OPA-19	580 – 1300	8	1 (12.50 %)	7 (87.50 %)
OPA-20	440 – 1300	4	3 (75.00 %)	1 (25.00 %)
Total	380 – 1550	46	18(39.13 %)	28(60.87 %)

*These bands are present in at least 95 % of total individuals investigated

gave the highest number of amplified bands (16 bands) while the lowest was found for primer OPA-20. Eighteen of these bands (39.13 %) were monomorphic and was present in at least 95 % of all individuals. Twenty-eight bands (60.87 %) were polymorphic (present in some individuals, absent in others) (Table 3). The percentages of polymorphic bands generated by primers OPA-11, OPA-14, OPA-18, OPA-19 and OPA-20 were 66.67, 50.00, 62.50, 87.50 and 25.00 %, respectively.

For individual samples in each population, the fish which were collected from Chiengrai, Sukhothai, Kanchanaburi, Chainat, Nakornpanom, Nongkhai, Suratthani, Songkhla and the hatchery population generated 34, 32, 31, 34, 35, 36, 37, 31 and 34 bands with the percentage of polymorphic bands of 26.47, 18.75, 25.81, 23.53, 31.43, 33.33, 54.05, 25.81 and 23.53 % respectively (Table 4). The results showed that the population with the highest number of polymorphic bands was Suratthani (54.05 %), while the lowest was the Sukhothai population (18.75 %).

The primer OPA-11 generated 6 scorable bands in all individuals of the 8 wild populations and the hatchery stock of *M. nemurus* in Thailand with molecular weights ranging from 750 to 1060 bp. Four of these zones (66.67 %) were polymorphic. Each population generated 2 to 4 bands (Table 4). The RAPD patterns of the OPA-11 primer showed 2 polymorphic bands in the Chainat population, and a polymorphic band each in the population from Chiengrai, Sukhothai, and Songkhla.

Primer OPA-14 produced 12 scorable bands with molecular sizes ranging from 460 to 1,550 bp. Half of these bands (50 %) were polymorphic. Each population showed 7 to 12 bands (Table 4) showing 7 polymorphic bands in the Suratthani population; 3 polymorphic bands in the Chiengrai and Nongkhai populations; 2 polymorphic bands in the Chainat, Nakornpanom and hatchery populations; and only one polymorphic band each in the Sukhothai, Kanchanaburi, and Songkhla populations.

Primer OPA-18 generated 16 scorable zones with molecular sizes ranging from 380 to 1,240 bp (*Fig. 3*). Ten of these bands (62.50 %) were polymorphic. Each population generated 11 to 13 bands (Table 4). There were 8 polymorphic

TABLE 4
Total number of bands, percentage of monomorphic and polymorphic bands found in 8 wild populations and a hatchery stock of *M. nemurus* from Thailand, which were generated through RAPD fingerprinting

Populations	No. of bands	Primers					Total	%
		OPA11	OPA14	OPA18	OPA19	OPA20		
Chiengrai	Total	3	12	12	4	3	34	100.00
	Monomorphic	2	9	9	3	2	25	73.53
	Polymorphic	1	3	3	1	1	9	26.47
Sukhothai	Total	3	11	11	4	3	32	100.00
	Monomorphic	2	10	7	4	3	26	81.25
	Polymorphic	1	1	4	0	0	6	18.75
Kanchanaburi	Total	3	8	11	6	3	31	100.00
	Monomorphic	3	7	8	2	3	23	74.19
	Polymorphic	0	1	3	4	0	8	25.81
Chainat	Total	4	11	12	4	3	34	100.00
	Monomorphic	2	9	8	4	3	26	76.47
	Polymorphic	2	2	4	0	0	8	23.53
Nakornpanom	Total	2	11	12	6	4	35	100.00
	Monomorphic	2	9	8	2	3	24	68.57
	Polymorphic	0	2	4	4	1	11	31.43
Nongkhai	Total	2	11	13	6	4	36	100.00
	Monomorphic	2	8	8	3	3	24	66.67
	Polymorphic	0	3	5	3	1	12	33.33
Suratthani	Total	3	11	13	6	4	37	100.00
	Monomorphic	3	4	5	2	3	17	45.95
	Polymorphic	0	7	8	4	1	20	54.05
Songkhla	Total	4	7	11	6	3	31	100.00
	Monomorphic	3	6	8	3	3	23	74.19
	Polymorphic	1	1	3	3	0	8	25.81
Hatchery	Total	3	9	13	6	3	34	100.00
	Monomorphic	3	7	9	4	3	26	76.47
	Polymorphic	0	2	4	2	0	8	23.53

bands in the Suratthani population; 5 polymorphic bands in the Nongkhai population; 4 polymorphic bands in the Sukhothai, Chainat, Nakornpanom and hatchery populations; and 3 polymorphic bands in the Kanchanaburi and Songkhla populations, respectively.

The primer OPA-19 generated 8 scorable zones in all individuals of *M. nemurus* with molecular sizes ranging from 580 to 1,300 bp. Seven of these zones (87.50 %) were polymorphic. Each population generated 4 to 6 bands (Table 4). The patterns of bands produced by the primer OPA-19 showed 4 polymorphic bands in the Kanchanaburi, Nakornpanom, and Suratthani populations, 3 polymorphic bands in the Nongkhai and Songkhla populations, 2 polymorphic bands in the hatchery stock, and a polymorphic band in the Chiengrai population. The populations from Chainat and Sukhothai showed identical patterns for all individual fish.

Primer OPA-20 produced 4 scorable bands with molecular sizes ranging from 440 to 1,300 bp. Only one band (25.00 %) in the Chiengrai, Nongkhai, Nakornpanom and Suratthani populations was polymorphic. The other populations showed identical patterns.

The RAPD patterns of all individuals of *M. nemurus* were used to calculate the genetic similarity index within and between populations. The average genetic similarity within populations based on RAPD patterns with 5 primers ranged from 0.822 to 0.960 (Table 5). The highest average similarity within population was found in the Chainat population with a value of 0.960 ± 0.020, while the lowest was found in the Suratthani population with a value of 0.822 ± 0.064.

The similarity index among populations based on RAPD patterns produced by 5 primers ranged from 0.711 to 0.913. The lowest genetic similarity was found between the Chainat and Suratthani populations with a value of 0.711. The highest genetic similarity was found between the Songkhla population and the hatchery stock with a value of 0.913.

When the similarity indices among populations were converted to genetic distances (*D*), they ranged from 0.087 between Songkhla population and the hatchery stock, to 0.289 between the Chainat and Suratthani populations (Table 6). The genetic distance values among populations of *M. nemurus* were used to construct a dendrogram using the unweighted pair-group method of clustering (UPGMA). The dendrogram, which depicts the relationship among populations of *M. nemurus*, is shown in *Fig. 4.*

DISCUSSION

The 5 primer produced 46 bands, of which 28 (60.87 %) were polymorphic. The results suggested that the highest percentage of polymorphic bands was found in the population from Suratthani (54.04 %), while the lowest was found in the Sukhothai population (18.75 %).

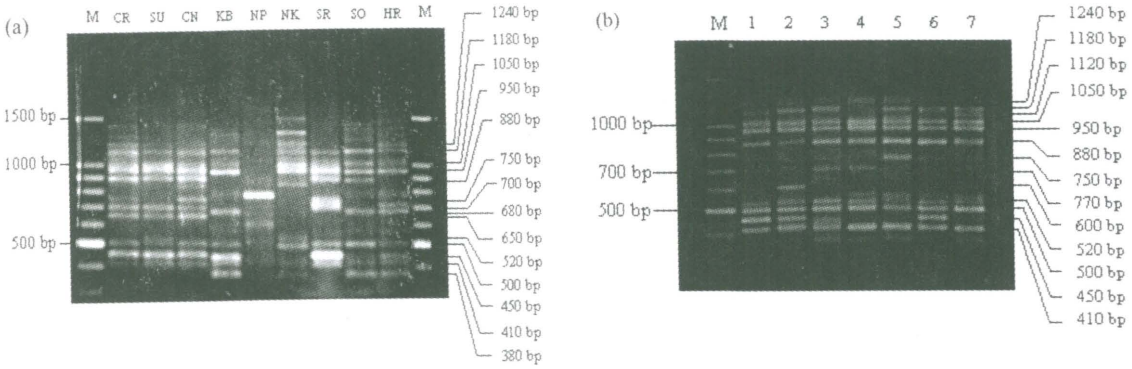

Fig. 3: RAPD patterns obtained from M. nemurus genotypes using primer OPA-18. Lane M: 100 bp DNA ladder. (a) The RAPD patterns were compared among different populations : CR=Chiengrai, SU=Sukhothai, CN=Chainat, KB=Kanchanaburi, NP=Nakornpanom, NK=Nongkhai, SR=Suratthani, SO=Songkhla, HR=Hatchery population. (b) Lanes 1-7: Individual samples collected from Nakornpanom (NP), north-eastern Thailand. Genetic distance (D)

TABLE 5
Estimated similarity index (*S*) within the eight wild populations and a hatchery stock of *M. nemurus* revealed by RAPD fingerprinting

Populations	Range of similarity index within population	Mean of similarity index within population
Chiengrai	0.868 – 1.000	0.948 ± 0.030
Sukhothai	0.818 – 1.000	0.929 ± 0.036
Kanchanaburi	0.857 – 1.000	0.946 ± 0.039
Chainat	0.909 – 1.000	0.960 ± 0.020
Nakornpanom	0.809 – 0.980	0.906 ± 0.036
Nongkhai	0.826 – 0.979	0.900 ± 0.030
Suratthani	0.634 – 0.978	0.822 ± 0.064
Songkhla	0.800 – 0.979	0.883 ± 0.037
Hatchery	0.800 – 0.981	0.898 ± 0.039

TABLE 6
Genetic distances among 8 wild populations and a hatchery stock based on Nei and Li (197) band sharing similarity index from RAPD markers

Population	CR	SU	KB	CN	NP	NK	SR	SO	HR
Chiengrai	****								
Sukhothai	0.091	****							
Kanchanaburi	0.139	0.139	****						
Chainat	0.139	0.145	0.092	****					
Nakornpanom	0.173	0.158	0.152	0.184	****				
Nongkhai	0.178	0.174	0.148	0.179	0.106	****			
Suratthani	0.266	0.283	0.252	0.289	0.230	0.255	****		
Songkhla	0.243	0.243	0.203	0.249	0.215	0.228	0.105	****	
Hatchery	0.258	0.249	0.188	0.236	0.228	0.229	0.140	0.087	****

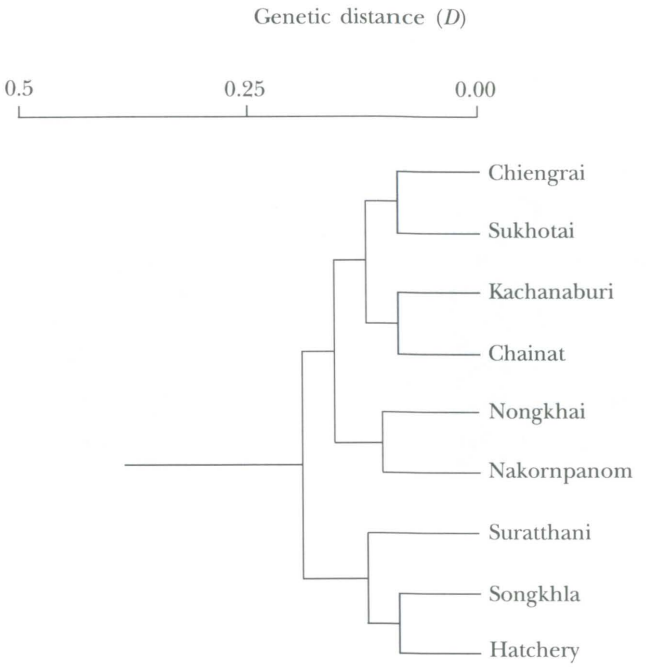

Fig. 4: UPGMA dendrogram constructed based on RAPD-PCR genetic distances among populations of M. nemurus in Thailand

The percentages of polymorphic loci revealed by RAPD fingerprinting were similar to the results obtained by Leesanga *et al.* 2000 using isozyme electrophoresis of 23 loci. They showed that the highest percentage of polymorphic loci was in the Suratthani population (43.48 %), and the lowest in the Sukhothai population with a value of 13.04 %. However, the percentage of polymorphic loci revealed by RAPD fingerprinting using 5 primers seemed higher than those revealed by isozyme analysis.

The estimated similarity index (*S*) within population ranged from 0.822 in the Suratthani population to 0.960 in the Chainat population. The values showed that there were a lot of genetic differences within populations from the south of Thailand (Suratthani, Songkhla and the hatchery stock) whereas less difference was found in the other populations. The results were in the same direction as the heterozygosity values revealed by isozyme electrophoresis of 23 loci (Leesanga *et al.* 2000).

The genetic similarities (*S*) among populations were converted to obtain the genetic distances (*D*), which ranged from 0.087 in the pair of Songkhla population and the hatchery

stock to 0.289 in the pair of Chainat and Suratthani populations (Table 6). The genetic distances among populations of *M. nemurus* in Thailand seemed lower than the genetic distances among populations of the fish reported in a neighboring country, Malaysia (Chong 1998; Foo 1998). However, the genetic distances in this study seemed in accordance with the results reported using isozyme electrophoresis of 23 loci (Leesanga *et al.* 2000). The genetic distance of *M. nemurus* from the Songkhla population to the hatchery stock located in Suratthani province is small (0.087). This may be because the fish from the hatchery population were transported to the Songkhla Inland Fisheries Station where they were used as broodstocks to produce fish fingerlings for release into natural water bodies or the reverse, that is the fish of the Songkhla population might have been transported and used as the broodstocks for the hatchery of the Suratthani Inland Fisheries Development Center. The highest genetic distance (0.289), which was found between the Chainat and Suratthani populations, should be supported by the morphometric data on characters between the two populations (Leesanga 2000).

The dendrogram depicts the genetic relationships among populations of *M. nemurus* (*Fig. 4*), which clustered into four groups according to their regions of origin. Samples from north (Chiengrai and Sukhothai), northeast (Nakornpanom and Nongkhai) and central Thailand (Kanchanaburi and Chainat) shared one major cluster with three subclasses whereas samples from south Thailand (Suratthani, Songkhla and the hatchery stock) were separated into another major cluster.

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