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Full Length Research Paper

Genetic diversity of Indonesia milkfish (*Chanos chanos*) using amplified fragment length polymorphism (AFLP) analysis

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Genetic diversity of milkfish (*Chanos chanos*) from Indonesia was investigated using amplified fragment length polymorphism (AFLP) markers. A total of 255 loci were detected by combination of seven primers from 130 individuals collected at seven locations. AFLP analysis provided useful information in determining genetic variation for milkfish from different locations. The proportion of polymorphic loci and heterozygosity among populations ranged from 12 to 72% and 0.041 to 0.187, respectively. Genetic differentiation (F_{ST}) and Nei's genetic distance among populations ranged from 0.214 to 0.732 and 0.016 to 0.302, respectively. Analysis of molecular variance supported significant differences between variance among populations when compared with variance within populations. The neighbor joining tree revealed four significantly distinct milkfish populations in Indonesian coastal waters. AFLP analyses may reveal historical patterns of milkfish genetic population structure in Indonesian coastal waters. The information derived from this study is useful to identify populations and categorize their population of origin to design a long term management program such as genetic improvement by selective breeding.

Key words: *Chanos chanos*, milkfish, genetic structure, Indonesia, amplified fragment length polymorphism (AFLP).

INTRODUCTION

The milkfish, *Chanos chanos* (Chanidae), is distributed widely in the Indo-Pacific region and inhabits subtropical and tropical areas, with longitude 140°E - 100°W and latitude 30°N - 30°S (Beveridge and Haylor, 1998). Milkfish is a euryhaline and migratory species, which grows and spawns in littoral waters (Huet and Timmermans, 1986). Sexually mature fish are found in coastal areas where salinity ranges from 0 to 70 ppt (Landau, 1992).

Milkfish has high nutrition content and is a valuable food fish in Southeast Asia for decades (Shiau, 2010). The first traditional milkfish aquaculture program started in Indonesia in the 1400s (Bardach et al., 1972) followed by Philippines (Bagarinao, 1998) and Taiwan (Chen and Hsu, 2006). Since then, considerable research and development efforts have resulted in reliable and consistent techniques for milkfish intensive mariculture and freshwater aquaculture (Bagarinao, 1998; Lee, 1998). However, limited information is available on genetic structure of milkfish populations (Su et al., 2002).

Development of molecular markers has made it possible to observe and exploit genetic variation in the nuclear genome (Liu and Cordes, 2004). Analysis of the nuclear genome is capable of providing unique genetic insights into fish populations in a wide range of applications (Verspoor, 1998). Among the variety of available genetic markers, amplified fragment length polymorphism (AFLP) markers (Vos et al., 1995) have become widely accepted in genetic structure studies. AFLP markers have been used for genetic variation studies in several species such as sturgeons, paddlefishes, black sea bream and masu salmon complex (Congiu et al., 2002; Ludwig, 2008; Hsu et al., 2008; Gwo et al., 2008). The major strength of the

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Abbreviations: AFLP, amplified fragment length polymorphism.

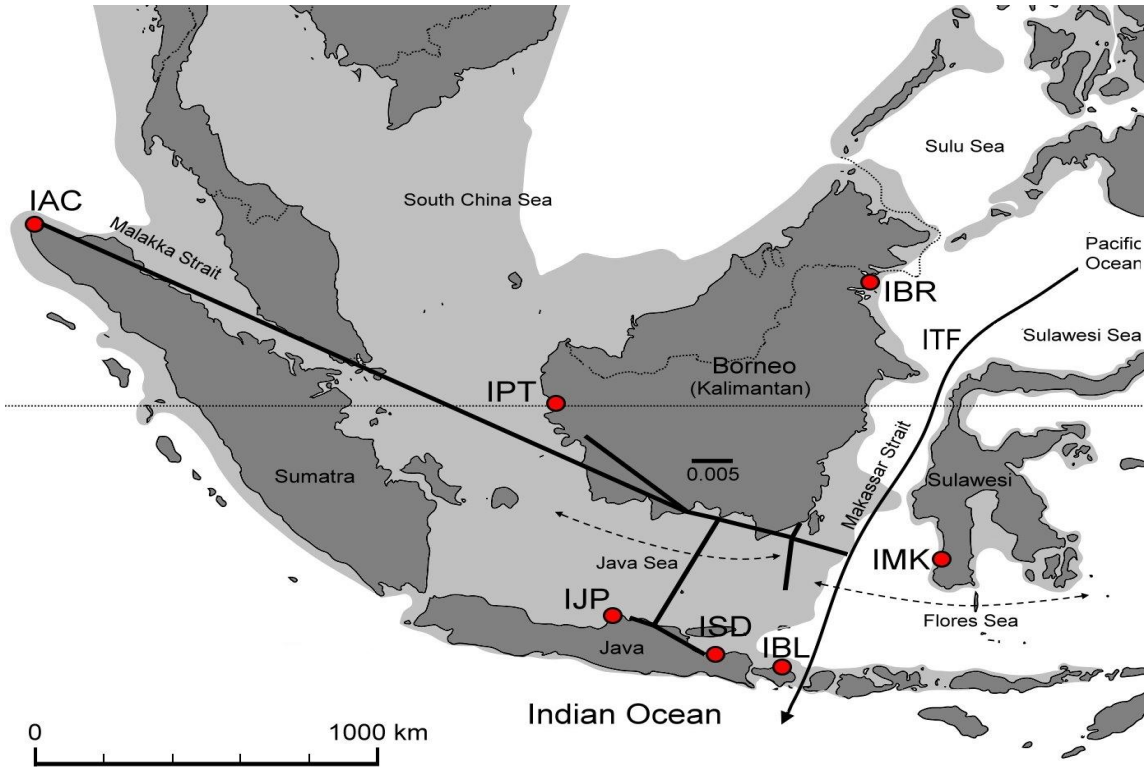

Figure 1. Map of the Indo-Malay archipelago with seven sampling sites (for abbreviations see Table 1) for milkfish, two major ocean currents and the NJ genetic relationship among these seven populations. Gray shading indicates coastal margins at 120 m below present sea level (Voris, 2000). Bootstrap support values (>50%) from 500 bootstrap replications are shown. ITF: Indonesian throughflow. ◄ – – ► (solid line) dominant current, (dashed line) seasonally reserving current.

AFLP markers includes their capability to detect a large number of population-specific polymorphism (Teletchea, 2009), high reproducibility due to high stringency of PCR and low cost per polymorphic marker (Liu, 2007). Moreover, AFLPs do not require any prior sequences information of the specimen that is under investigation. Hence, AFLP markers are applicable to fish species for which sequence information is not available.

Several studies relating to the population structure of milkfish have been reported (Smith, 1978; Winans, 1980, 1985; Villaluz and MacCrimmon, 1988; Ravago et al., 2002; Ravago-Gotanco and Juinio-Meñez, 2004), but no information comparing milkfish populations in Indonesia is available. For the sustainable development of the milkfish industry, understanding of the genetic structure of milkfish populations is urgently needed. This study aimed to investigate the genetic diversity of milkfish populations from Indonesian coastal waters using AFLP markers.

MATERIALS AND METHODS

Sample collection and DNA extraction

A total of 130 milkfish were collected from seven coastal locations across Indonesia (Figure 1 and Table 1). Milkfish fin clipped samples were preserved in 95% ethanol before DNA extraction. Genomic DNA was extracted from fin clips (15 to 25 mg) using Proteinase-K (Amresco,USA) digestion and AxyPrep Multisource Genomic DNA Miniprep Kit (Axygen Bioscience, USA) following the manufacture's methods. DNA concentration was measured with Nanodrop (Nanodrop, USA). The quality of extracted DNA was assessed by 1.0% agarose electrophoresis (Agarose I, Amresco, USA) with loading dye mixture with ethidium bromide (EZ-Vision TM Three, Amresco, USA) and DNA ladder (Bio-100bp, Protech, Taiwan) in a UV light box (A UVB-1, Model UVB-101) and documented using an EZ-Catcher Camera (EZC-2002).

Development of AFLP marker

Procedures of AFLP analysis were essentially based on Vos et al. (1995). Genomic DNA templates for AFLP reactions were generated by double restriction enzyme digest and ligation. Initially, about 100 ng genomic DNA in TE buffer of each sample was digested with 12 U/µl *Tru*91 (Promega, USA) for 3 h at 37°C and *Eco*RI (Promega, USA) for 3 h at 37°C and 15 min at 75 °C. To generate DNA templates for subsequent PCR amplification, the digested DNA fragments were ligated with 5 µM *Eco*RI and 25 µM *Tru*91 adapters in a reaction mixture containing 3 U/µl T4 DNA Ligase (Promega, USA) and 10x Ligase Buffer (Promega, USA) for 3 h at 37°C and 15 min at 75°C.

The PCR reaction was conducted using Eppendorf Mastercycle Gradient (USA) in a 20 µl reaction containing 4 µl 5x Flexi buffer, 1.6 µl $MgCl_2$ (25 mM), 5 U Taq Polymerase DNA (Promega, USA)

Table 1. Population location, abbreviation and sample size of milkfish (*C.chanos*) populations.

Population location	Abbreviation	Sample size
Pontianak, Borneo (Kalimantan) Island	IPT	20
Berau, Borneo (Kalimantan) Island	IBR	20
Makassar, Sulawesi Island	IMK	15
Bali, Bali Island	IBL	15
Sidoarjo, Java Island	ISD	20
Jepara, Java Island	IJP	20
Aceh, Sumatra Island	IAC	20

and 1 µl dNTPs (2mM) (Protech, Taiwan). The amplification reaction was performed at 95°C for 2 min, denaturation at 94°C for 30 s, annealing at 53°C for 30 s for 30 cycles and extension at 72°C for 1 min. The 20 µl product was diluted 5 fold with distilled water and used as templates for subsequent PCR selective amplification.

Selective amplification was conducted using Eppendorf Mastercycle Gradient (USA) in 10 µl reaction containing 4 µl 5x Flexi buffer, 1.6 µl $MgCl_2$ (25 mM), 5 U Taq Polymerase DNA (Promega, USA) and 1 µl dNTPs (2 mM) (Protech, Taiwan). The amplification reaction was performed with touchdown PCR begin at 95°C for 2 min, 65°C for 30 s, 72°C for 1 min, 10 cycles at 94°C for 2 min, 65°C (decreasing 1°C each cycle) for 30 s, and 72°C for 1 min. The last reaction was performed at 94°C for 30 s, 56°C for 30 s; 72°C for 1 min with 30 cycles and final extension of 72°C for 5 min. The selective amplification was performed using seven pairs of primers (*Eco*RI-ACT with *Tru*91-CGG, *Eco*RI-ATC with *Tru*91-CGT, *Eco*RI-ATA with *Tru*91-CGC, *Eco*RI-AGA with *Tru*91-CAA, *Eco*RI-ATG with *Tru*91-CAA, *Eco*RI-AGT with *Tru*91-CCG and *Eco*RI-ATC with *Tru*91-CGG).

Electrophoresis and silver staining

Fingerprinting patterns were visualized on 8% denaturing polyacrylamide gel electrophoresis (PAGE) using the silver staining method. The PCR products were mixed with equal volumes of 6X loading buffer (Protech, Taiwan). The mixture of the products were denatured at 95°C for 3 min and immersed immediately into ice. Samples were cooled on ice for 10 min prior to loading on 8% PAGE prepared as follows: 50 ml of acrylamide 19:1/40% (Amresco, USA), 120 g of urea (Amresco, USA) and 50 ml 5 x TBE. This PAGE was mixed with 16 µl TEMED (Amresco, USA) and 320 µl of 10% ammonium per sulfate (APS) (Amresco, USA).

PAGE pre electrophoreses were run at 1800 V (Bio-Rad PowerPac HV Power Supply, USA) for 3 h in a ATTO-AE6155 (ATTO Corporation, Japan) DNA sequencing cell, each well loaded with 1 µl sample and 0.8 µl 10 bp ladder (Promega, USA). After electrophoresis, the gel was fixed in a 1% acetic acid solution (Nacalai Tesque, Japan) for 1 h. The gel was rinsed in distilled water and stained with a mixture of 1 g of silver nitrate, 200 µl $Na_2S_2O_3$ (Nacalai Tesque, Japan) and 1.5 ml of formaldehyde solution (Hayashi Pure Chemical, Japan) in 1 L distilled water for 1 h and 20 min. The final gel was stained with 5 g NaOH (Nacalai Tesque, Japan) and 1.5 ml formaldehyde solution in 1 L distilled water until bands visualized and reached desirable intensity. Band size was estimated using a standard ladder and analyzed using an Image Analysis System (HP ScanJet 5370C).

Data analysis

AFLP bands were scored for all seven pairs of primer combination with presence (1) or absence (0) and transformed into a 1/0 binary character matrix. Estimates of similarity were based on the number of shared amplification products. We assumed that each band position corresponded to loci among individual. Proportion of polymorphic loci and heterozygosity (*H*) among populations were calculated by AFLP data analyzer version 1.3 and GenAlEx 6.3 (Peakall and Smouse, 2006). Nei genetic distances (*D*) and genetic differentiation (F_{ST}) were calculated using AFLP-SURV 1.0 (Vekemans et al., 2002) and Arlequin 3.5.1.2 (Excoffier et al., 2005).

Genetic differentiation within and among populations were estimated using the analysis molecular of variance (AMOVA) software package in GenAlEx 6.3 (Peakall and Smouse, 2006). A neighbor joining (NJ) phylogenetic tree was estimated to give an overview of the genetic relationship among populations using PHYLIP 3.68 (Felsenstein, 2008) and MEGA4 (Tamura et al., 2007).

RESULTS

The proportions of polymorphic loci provide a good measure of genetic variations within and among populations of milkfish. A total of 255 loci were detected by the seven primer combination in seven populations, 138 (54.53%) of which were polymorphic (Table 2). The average number of total loci scored per primer pair was 36.43, ranging from 27 to 48 loci. The average number of polymorphic loci scored per primer was 19.71 loci, ranging from 16 to 27 loci (Table 2). The population with the highest proportion of polymorphic loci (71.76%) and number of polymorphic loci (183) was Aceh (IAC), whereas that with the lowest was Makassar (IMK) population in which the proportion of polymorphic loci and number of polymorphic loci was 12.55% and 32, respectively (Table 3). The population with the highest heterozygosity (H = 0.187) was Aceh (IAC) and the Sidoarjo (ISD) population had the lowest H value (0.041) (Table 3).

Results of the AMOVA showed that most variation was contributed by variance among populations 17.328 (60%) when compared with variance within populations 11.764 (40%), suggesting that a high genetic variation among populations. Furthermore, overall genetic differentiation among milkfish from the 7 populations was significant, suggesting significant genetic differentiation among localities. Moreover, pairwise F_{ST} values among populations were also high and significant, ranging from 0.214 to 0.732 (Table 4). These analyses indicated that several

Table 2. AFLP primer pairs used in this study and approximate number of amplification products per primer pair of milkfish (*C. chanos*) populations.

Primer pair	Total Loci	Monomorphic loci		Polymorphic loci	
		Number	Proportion (%)	Number	Proportion (%)
E-ACT/M-CGG	30	12	40.00	18	60.00
E-ATC/M-CGT	42	20	47.62	22	52.38
E-ATA/M-CGC	27	11	40.74	16	59.26
E-AGA/M-CAA	40	22	55.00	18	45.00
E-ATG/M-CAA	48	21	43.75	27	56.25
E-AGT/M-CCG	35	16	45.71	19	54.29
E-ATC/M-CGG	33	15	45.45	18	54.55
Total	255	117	-	138	-

Table 3. Comparison of polymorphic loci and heterozygosity among milkfish (*C. chanos*) populations.

Population	Polymorphic loci		Heterozygosity (H)
	Number	Proportion (%)	
IPT	109	42.75	0.056
IBL	35	13.73	0.055
IMK	32	12.55	0.104
IBR	33	12.94	0.042
ISD	52	20.39	0.041
IJP	63	24.71	0.046
IAC	183	71.76	0.187
Total	507	-	-

distinct populations of the milkfish existed in Indonesian coastal waters. The lowest genetic difference among populations existed in Sidoarjo (ISD) and Jepara (IJP) (F_{ST} = 0.214), whereas the difference between Makassar (IMK) and Sidoarjo (ISD) was the largest (F_{ST} = 0.732). Nei's genetic distance analysis also suggested that populations in Sidoarjo (ISD) and Jepara (IJP) were the most similar genetically (D = 0.016), whereas the populations from Makassar (IMK) and Aceh (IAC) were the most different genetically (D = 0.302) (Table 4). The NJ tree generated from the loci profiles of AFLP showed four geographic groups: populations from Bali (IBL), Makassar (IMK) and Berau (IBR) as group 1, Pontianak (IPT) population as group 2, Sidoarjo (ISD) and Jepara (IJP) populations as group 3 and Aceh (IAC) population as group 4 (Figure 1).

DISCUSSION

This study is the first attempt to compare genetic structure among milkfish populations from Indonesian coastal waters using AFLP markers. Previously, milkfish genetic variation was investigated using isozymes (Winans, 1980) and mitochondrial restriction fragment length polymorphism (RFLP) markers (Ravago-Gotanco and Juinio-Meñez, 2004). The reported values of proportion of polymorphic loci, heterozygosity (H) and genetic differentiation (F_{ST}) were all considerably lower than the results of this study. The differences are likely due to the ability of AFLP markers to detect more loci and higher levels of polymorphism than those of isozymes and mitochondria RFLP markers. One of the benefits of AFLP over other molecular techniques is its sensitivity for stock identification without any upfront knowledge of the species' genome, providing a large number of independent markers that can be rapidly surveyed (Liu and Cordes, 2004; Gwo et al., 2008).

The milkfish is a widely distributed, euryhaline teleost occurring on continental shelves and islands with coral-reef areas throughout the entire tropical Indo-Pacific Ocean (Bagarinao, 1994; Beveridge and Haylor, 1998; Martinez et al., 2006; Liao and Leaño, 2010). Adults spawn offshore (about 30 Km) in clear shallow (<200 m depth) sea waters over sandy or coral bottoms near the surface (Bagarinao, 1994; Lee, 1998). The pelagic larvae appear in the surf zone, moving inshore and settling in shallow water nurseries (mangrove swamps, estuaries and sometimes lakes) for two to three weeks and returning to the sea to mature and reproduce (Bagarinao, 1994; Lee, 1998). However, adult milkfish are rarely captured in the high seas. Pelagic marine fishes usually

Table 4. Pairwise matrix F_{ST} (above diagonal) and Nei genetic distance (*D*) (below diagonal) of milkfish (*C. chanos*) populations.

Population	IPT	IBL	IMK	IBR	ISD	IJP	IAC
IPT		0.502*	0.472*	0.451*	0.608*	0.582*	0.521*
IBL	0.100		0.311*	0.302*	0.692*	0.662*	0.645*
IMK	0.082	0.023		0.300*	0.732*	0.710*	0.660*
IBR	0.078	0.023	0.021		0.696*	0.669*	0.641*
ISD	0.165	0.140	0.158	0.137		0.214*	0.602*
IJP	0.145	0.119	0.139	0.119	0.016		0.610*
IAC	0.228	0.293	0.302	0.280	0.244	0.252	

]
*Significant P <f 0.05. IPT, Pontianak; IBL, Bali; IMK, Makassar; IBR, Berau; ISD, Sidoarjo; IJP, Jepara; IAC, Aceh.

show low levels of genetic differentiation among geographic regions because of high dispersal potential at egg, larval and adult stages (Lin et al., 2009). However, this study provided the molecular evidence for the existence of separate milkfish populations (stocks) in Indonesian coastal waters. The AMOVA analysis also supported genetic differentiation among Indonesia milkfish populations. Four geographic groups were revealed by NJ tree based on Nei's genetic distance (Figure 1), suggesting that milkfish in Indonesian coastal waters are not a single panmictic population. The result was consistent with the previous study based on RFLP analysis of a portion of the mitochondrial control region (Ravago-Gotanco and Juinio-Meñez, 2004), supporting two genetically distinct stocks in Philippine milkfish. Lee and Liu (2010) also reported that the natural milkfish stocks are isolated and do not interchange between different locations, even when as close as the islands of Oahu and Hawaii in the Hawaiian Island Chain. The milkfish genetic population structure is distinctly diverse in the Pacific, as demonstrated by morphological and biochemical studies (Winans, 1980, 1985). The NJ pattern is meaningful from a geographical perspective. The four genetically differentiated geographic populations represent the Malacca Strait population (Aceh), the South China Sea population (Pontianak), the Java Sea population (Jepara and Sidoarjo) and the Makassar Strait and Indonesian throughflow population (Berau, Bali and Makassar) (Figure 1).

Division of Indo-Malay archipelago populations of milkfish along the Wallace's Line in this study was supported by both AMOVA and NJ of AFLP data. Nuclear markers (allozymes, AFLP) and morphological characters may reveal historical patterns of genetic population structure (Williams et al., 2002). The complex genetic population structure and pattern of connectivity we found here may be attributed to past geological history of the Indo-Malay archipelago. Indonesia straddles Wallace's Line and bisects the Pacific and Indian Oceans (Voris, 2000; Williams et al., 2002). The Indo-Malay archipelago in the Indonesian region is both geographically and hydrologically complex. At least two main ocean currents influence the distribution of milkfish larva within the archipelago. The first is the Indonesian throughflow, with a velocity of 1 m/s flowing from the Pacific Ocean, passing through the Makassar and Lombok Straits to the India Ocean (Barber et al., 2000; Voris, 2000). The second is the seasonally reversing east-west current that flows between the Java and Flores Seas at a velocity of 0.75 m/s (Barber et al., 2000; Voris, 2000). In our NJ analysis, the grouping of group 1 (Bali, Makassar and Berau) and group 3 (Sidoarjo and Jepara) strongly suggested that these two ocean currents play a major role in generating the present genetic structure. Ocean currents and other barriers might also limit gene flow. Repeated fluctuations of sea levels in this topographically complex region could have favored the geographic isolation of several inland seas during the Pleistocene glacial age, leading to the genetic differentiation of the populations they harbored. Variation in habitat and topography has proved to be substantial barriers to gene flow in other species (Barber et al., 2000; Williams et al., 2002; Timm and Kochzius, 2008). Other variables such as larval behavior, the duration of the pelagic stages or settlement habitat choice influence the dispersal and gene flow of pelagic reef fishes as well (Timm and Kochzius, 2008). This vicariance is generally accepted to be the origin of a major phylogeographical break between Pacific and Indian populations of several marine species (Barber et al., 2000; Williams et al., 2002). Although, genetic analyses of marine species across the Indo-Malay region are still few in number, similar results and conclusions, a genetic break, have been reported in fish, giant clam and shrimp (Barber et al., 2000; Williams et al., 2002; Timm and Kochzius, 2008).

Milkfish hatchery owners obtain their brood populations from the wild or other breeders in other locations (Liao, 1991; Sudrajat and Sugama, 2010). Brood populations are usually derived from unknown genetic background or limited genetic information and from minimal number of brood populations. Poor brood population management commonly leads to further loss of genetic variation (genetic drift and inbreeding effect) (Allendorf and Ryman, 1988), especially in high fecundity species such as

milkfish, so hatcheries tend to use fewer numbers of brood populations to meet production target. Milkfish genetic structure information derived from this study will support and provide guidelines to identify populations and categorize their population of origin to design a long term management program such as genetic improvement by selective breeding.

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