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Genetic Variation among Populations of Pla-Mong Fish (*Pangasuis bocourti* Sauvage 1880) of the Mae Kong River in Northeast Thailand

[1]T. Champasri, [2]W. Jiwyam, [3]Ch. Budriang and [1]S. Charoenwattanasak
[1]Department of Fisheries, Faculty of Agriculture, Khon Kaen University, Khon Kaen 40002, Thailand
[2]School of Fisheries, Nong Khai Regional Campus, Khon Kaen University, Nong Khai 43000, Thailand
[3]Department of Bio-chemistry, Khon Kaen University, Khon Kaen 40002, Thailand

Abstract: This study was carried out at the Department of Fisheries, Faculty of Agriculture, Khon Kaen University, Khon Kaen, Thailand during April to September 2007 to determine DNA patterns of Pla-mong fish (*Pangasuis bocourti* Sauvage 1880) with the use of RAPD-PCR amplification. One hundred twenty individual fish samples were harvested from four locations along the Mae Kong River, i.e., Nongkhai, Nakornphanom, Mukdaharn and Ubon Ratchatani provinces, each location has thirty individual fish samples and the four locations were used as treatments and thirty individual fish of each location were used as replications. Sixteen RAPD decamer primers from three kits of Operon Technologies were subjected to a preliminary test and only seven decamer primers were suited most for PCR amplification. The results on both similarity correlation coefficients and genetic distances revealed that the fish of Pla-mong of the Mae Kong River could be divided into two groups, i.e., the first group included the fish harvested from Nongkhai and Nakornphanom provinces with their genetic values ranged from 0.20 to 0.36 and the second group included the harvested fish from Mukdaharn and Nakornphanom provinces with their genetic values ranged from 0.20 to 0.44.

Key words: Decamer primers, DNA bands, genetic distance, Pla-mong fish

INTRODUCTION

The production of freshwater fish of various species being harvested annually from rivers and wetlands has its significant role in the Thai economy since a large number of the population depended largely on fish proteins for their daily diets due to low market price when compared with other sources of proteins such as beef, pork and others. Pla-mong, a Thai common name for this freshwater fish of *Pangasuisbocourti* Sauvage 1880 has been widely known among the Thai consumers. This type of fish could be found in many large rivers in Southeast Asia such as the Mae Kong River, the Chao Phraya River and some other inland smaller rivers, e.g., the Mun River and the Chi River and many others those located in Northeast Thailand. The good taste flesh of this fish species has been accepted worldwide among the consumers, thus the consumers, particularly the Thai people demanded some large amount of this fish species annually. The high demand for this fish around the globe has been increased from time to time, especially in Europe and America (Jones and Young, 1996). For American and European countries, the filet forms are mostly demanded by the markets hence the prices have considerably increased. In Thailand an individual fish weighed 0.7-1.0 kg each could cost up to 50 baht, whilst individual fish, each weighed 1.5 to 2.0 kg could cost up to 150 baht, which is nearly 5 US Dollars (Prasertwattana *et al*., 2003). Thailand exported fresh filets of this type of fish in the 1999 and 2004 with values of 20,000 and 120,000 metric tons, respectively. However, the quality of the filets was only at a moderate level due to the decline in color, which was not purely white (Imsilp, 2005).

This type of fish belonged to Pangasiidae family and within the family they consisted of four genera, i.e., *Heliphagus*, *Pangasianodon*, *Pteropangasius* and *Pangasius*. Some published data revealed that these families of fish are abundantly available in both the Mae Kong and Chao Phraya rivers (Roberts, 1989; Roberts and Vidthayanon, 1991; Vidthayanon and Rungtongbaisuree, 1993). The study of Sirikul and Prarom (1995) and Champasri and Jeiwyam (2002) reported that this type of fish mostly habited in current water with high amount of available oxygen content. They normally lay eggs yearly commencing from the months of May to June. With a tremendous demand for fleshes of this type of fish in domestic and overseas markets, thus the objectives of this investigation included the feasibility in culturing this

Corresponding Author: Thongchai Champasri, Department of Fisheries, Faculty of Agriculture, Khon Kaen University, Khon Kaen 40002, Thailand

fish at a large scale for commercial unitization and to search for more information on how genetic variation could occur among the fish populations in their habitation in Northeast Thailand.

Nowadays, it is well justified that the advancement in biological sciences have contributed largely to science world, thus a number of research techniques had been innovated and applied for use in many investigations. The high progress on DNA-based markers development had made it possible to identify genetic variation in many important animals, particularly the aquatic animals. The use of Polymerase Chain Reaction (PCR) method has been used along with many techniques such as Restriction Fragment Length Polymorphism (RFLP), Randomly Amplified Polymorphic DNA (RAPD) along with Polymerase Chain Reaction (PCR), Amplified Fragment Length Polymorphism (AFLP), Microsatellite, Single Nucleotide Polymorphism (SNP) and Expressed Sequence Tag (EST) markers (Baradakci and Skibinski, 1994; Stepien and Kocher, 1997; Callejas and Ochando, 2001; Bártfai *et al*., 2003; Liu and Cordes, 2004). For this study, an RAPD-PCR method was chosen and the objectives of this research include: (1) the search for more information on gene variation in the populations of Pla-mong fish of the Mae Kong River. (2) Pla-mong fish has its important role in the Thai economy, thus the attained information could be used as a foundation for further academic investigations. In the near future, Thailand could possibly be able to increase the annual production of this type of fish for domestic and overseas markets.

MATERIALS AND METHODS

This RAPD-PCR study was carried out at the Faculty of Agriculture, Khon Kaen University, Khon Kaen, Thailand during the months from April to September 2007. Four fish locations (provinces) were chosen as treatments, i.e., Nongkhai, Nakornphanom, Mukdaharn and Ubon Ratchatani where the four sites are located along the Mae Kong River starting from Nongkhai up to Ubon Ratchatani provinces. Thirty-individual fish with a range of weight between 1-3 kg for individual fish from each location were randomly chosen and immediately dissected for approximately 10 g of flesh samples and then kept in liquid nitrogen containers ready for DNA extraction in laboratory. The thirty fish samples from each location were used as replications. The genomic DNA extraction was carried out with the use of fish muscle tissues (skeleton muscle) where the DNA isolation kit protocol (Gentra systems Inc., 2000) was used. For each sample tested, 5 mg of fish muscle tissues were placed into 1.5 mL microcentrifuge tube and 300 μL of Cell Lysis Solution was added. Tubes of samples were incubated in water-bath at 65°C for 15 min prior to the adding up of 1.5 μL of 20 mg mL^{-1} Proteinase K Solution. The lysate was mixed by inverting at least 25 times until it thoroughly mixed and then incubated at 55°C overnight. After particulate tissue had dissolved, 1.5 μL of 4 mg mL^{-1} RNase A solution was added to the cell lysate to eliminate RNA. The sample was mixed by inverting 25 times followed by an incubation process at 37°C for 30 min. The sample was cooled down to room temperature before adding 100 μL of Protein Precipitation Solution in order to precipitate the proteins. The tubes of samples were vigorously processed at a high speed for 20 sec as to mix the Protein Precipitation Solution uniformly with the cell lysate. The precipitated proteins were separated by the centrifugation at 16,000x g for 3 min. The supernatant containing the DNA was transferred into a cleaned microcentrifuge tube containing 300 μL (100%) isopropanol and mixed by inverting gently 50 times. Tube was centrifuged again for 1 min and then a white DNA pellet was visible. The supernatant in upper level was poured off and then drained out with a cleaned absorbent paper. To wash the DNA pellet, 300 μL of (70%) ethanol was added. Tube was inverted several times, centrifuged at 16,000x g for 1 min and carefully poured off the ethanol. At this step, pellet may be loosed so ethanol was pouring slowly. The tube was inverted, drained on cleaned absorbent paper and allowed to air-dry for 30 min. Fifty microlitre of DNA Hydration Solution was added to dissolve the DNA. The attained DNA solution was diluted with doubled distilled water to attain 50 ng μL^{-1} as a DNA reserved stock before long-term storage at -20°C. DNA samples of individual fish species were aliqouted and diluted to 25 ng μL^{-1} with doubled distilled water and were then used as the template for RAPD-PCR amplification. Before RAPD-PCR amplification was taken place, a selection for suitable decamer primers was carried out. Three sets of decamer primers were chosen, i.e., OPA01-20, OPC01-20 and OPY01-20 (Operon Technologies Inc., Alameda, USA). There were seven decamer primers suited most for DNA multiplication. They include OPA02 (5'-TGCCGAGCTG-3'), OPA12 (TCGGCGATAG-3'), OPA20 (5'-GTTGCGATCC-3'), OPC05 (5'-GATGACCGCC-3'), OPC08 (5'-TGGACCGGTG-3'), OPC14 (5'-TGCGTGCTTG-3') and OPY07 (5'-AGAG CCGTCA-3'). For PCR steps in multiplying DNA to be followed include 1x Taq buffer, 0.2 mM of each dNTPs (dATP, dTTP, dCTP, dGTP), 2.0 mM $MgCl_2$, 10 pmol primer, 25 ng genomic DNA and 1.0 Unit of Ampli Taq DNA polymerase. The amplification was carried out in a Thermal Cycler Machine (Corbett Research, Netherlands) in which reaction was initially preheated at 94°C for 2 min

followed by 43 cycles of denaturation at 94°C for 1 min, annealing at 38°C for 1 min and extension at 72°C for 5 min prior to finally extended at 72°C for 10 min to allow a complete extension of all amplified fragments.

Approximately 10 μL of RAPD-PCR amplified products plus 3 μL of 1x loading dye (25% bromphenol blue, 25% xylene cyanol and 40% sucrose) were loaded in a 2.0% agarose gel and then subjected to electrophoresis in 1x TBE buffer (89 mM Tris-HCl, 89 mM boric acid and 2 mM EDTA, pH 8.0) from cathode to anode at 100 volts until the bromophenol blue marker migrated almost out of the gel. After the finishing of the process, the gels were stained with ethidium bromide (0.5 μg mL^{-1}) for 10 min prior to de-staining to remove an excessive ethidium bromide by submerging in an excessive amount of distilled water for 15 min. The nucleic acid bands were visualized under UV transillumination in Gel-Document (being attached with a camera) provided with GeneSnap software (SYNGENE, The United Kingdom) and then photographic action was taken place. GeneSnap software was used to aid in recording the DNA bands. The score of 1 represented the presence of DNA band, whist the score of 0 was used for the absence of any DNA band. The software was then used to transform the reading scores of DNA bands to XLS file. The attained file was subjected to NTSYS-pc 2.10 programme (Rohlf, 2000) for further calculations to attain similarity index values and genetic distances. Finally, both similarity index values and genetic distances were calculated further through a SAS Computer Programme (SAS Institute Inc., 1998).

RESULTS

DNA concentration, quality and polymorphic bands: The results carried out in the laboratory revealed that the extracted DNA concentration of the fish samples ranged from 67 to 2,342 ng with their DNA purification quality level ranged from 1.43 to 1.82. The DNA bands were attained with the RAPD-PCR technique where seven commercially decamer primers were used. After an increase in the amount of DNA to match the decamer primers with the applied technique, it revealed that DNA bands increased to a maximum value of 3,840 bands and the band width of DNA ranged from 200 to 1,000 based pairs with percentages of polymorphic bands ranged from 38.43 to 56.82. The whole lot of DNA ladders could not be accommodated in this study, thus only some DNA ladders are available in Fig. 1a and b.

Similarity coefficient indices and genetic distances: The attained DNA data of the fish were calculated and a dendrogram figure was established, this was aiming to provide evidences on similarity coefficient indices with the use of the Un-weighted Pair Group Method of Analysis (UPGMA). The results revealed that there were two groups of fish found, i.e., the fish belong to the first group included the fish harvested from both Nongkhai and Nakornphanom provinces and the second group included the fish from both Mukdaharn and Ubon Ratchatani provinces (Fig. 2). The results on genetic distances showed that the first and second groups of fish possessed genetic distances between 0.20-0.36 and between 0.20-0.44, respectively (Table 1).

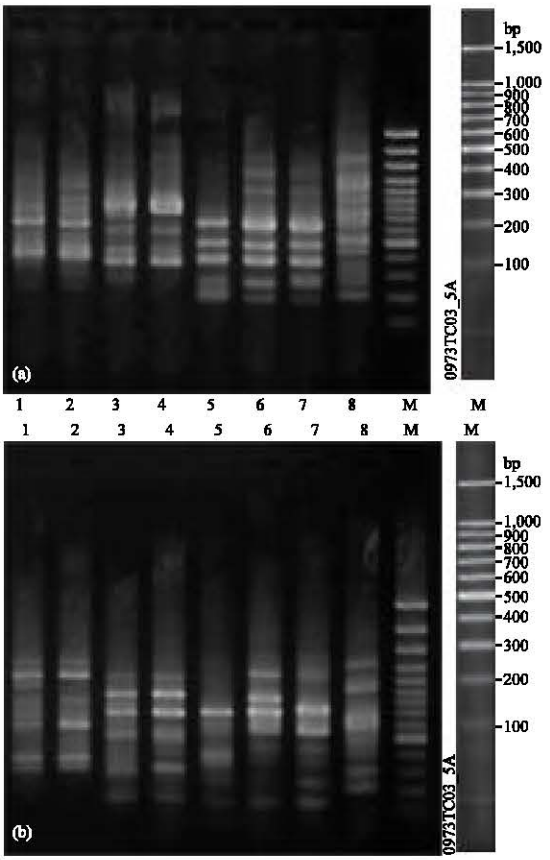

Fig. 1: (a) The Illustration of DNA bands of Pla-mong fish where it derived from an increase in amount of DNA with the use of an OPA02 decamer primer; No. 1-2 derived from Nongkhai, 3-4 from Nakornphanom, 5-6 from Mukdaharn, 7-8 from Ubon Ratchatani. M : Marker (100 bp DNA ladder); (b) DNA bands derived from the use of an OPC08 decamer primer, 1-2 from Nongkhai, 3-4 from Nakornphanom, 5-6 from Mukdaharn, 7-8 from Ubon Ratchatani, M : Marker (100 bp DNA ladder)

Amongst the different sources of protein to feed the world population, fleshes of the various kinds of fish play

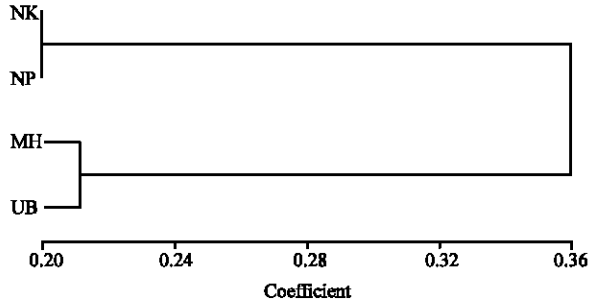

Fig. 2: A dendrogram figure illustrating correlation coefficient values separated Pla-mong fish into two groups, i.e., the first group included the fish from both Nongkhai (NK) and Nakornphanom (NP) and the second group included the fish from both Mukdaharn (MH) and Ubon Ratchatani (UB) provinces, Northeast Thailand

Table 1: Genetic distances among the fish of Pla-mong harvested from four locations of the Mae Kong River, i.e., Nongkhai (NK), Nakomphanom (NP), Mukdaharn (MH) and Ubon Ratchatani (UB) provinces, Northeast Thailand

Locations	NK	NP	MH	UB
NK	0.00			
NP	0.20	0.00		
MH	0.24	0.33	0.00	
UB	0.44	0.42	0.20	0.00

its significant role in national economy in most countries around the globe, since the share of fish protein to the total food expenditure are higher among lower income groups, where the groups depended largely on fish protein (FAO, 1999; Dey, 2000; Dey *et al*., 2004), hence Williams (1996) stated that poor man's protein comes from fish, a primary source of protein among relatively poor household units. In Southeast Asian countries about 45% of protein intake derived from fish production (Prein and Ahmed, 2000). An annual amount of fish production in Thailand those derive from both freshwaters of natural habitations and fish culture has its important role in the Thai economy. For Pla-mong fish (*Pangasuis bocourti* Sauvage 1880) alone, in the year 2004, apart from domestic consumption, an exported record overseas reached a value of 120,000 metric tons with an approximate value exceeded five hundred millions US Dollars (Imsilp, 2005). This type of fish species has its significant role among most household units of villagers of the nearby locations along the Mae Kong River since large numbers of the villagers have engaged in fisheries as an alternative career for their daily income and food protein. Thus there is an urgent need to learn more of this type of fish since a high demand for inland and overseas has been increasing from time to time, particularly the countries in the continental of Europe and the USA (Jones and Young, 1996).

In Thailand, there has been some progress in multiplying a certain amount of Pla-mong fish by artificial insemination, e.g., Sirikul and Prarom (1995) and Singsee (2005) reported that their artificial insemination processes with the use of Pla-mong fish were successfully attained and they were able to release a large number of the survived fish offspring to some inland rivers apart from being cultured in cages by villagers. Thus in the near future villagers along the river banks of some important rivers could possibly increase their annual fish production by increasing number of cages along the river's banks where current water continues to supply adequate amount of oxygen for the fish. Nevertheless, if artificial insemination is carried out without adequate genetic information of the fish species then some damages may be occurred within the species of the fish. Therefore, the search for more information on genomic traits of any economically fish are of imperative value, i.e., the attained results of work could provide adequate information for appropriate breeding activities and at the same time scientists could conserve any distinguish fish to remain its true to type species. Ngoychansri and Petcharatana (2005) reported that a population of fish of Pla-mong at a rate of 50 individual fish m^{-3} being cultured in cages in Thailand gave the highest growth rate day^{-1} (live weight of 2.12 g day^{-1}), hence in the near future it is possible that villagers could possibly pay more attention in culturing this type of fish at a large scale along the banks of the Mun River and also the Chi River, apart from the Mae Kong River. From this investigation, according to genomic traits of the fish, it was found that the fish of Pla-mong of the Mae Kong River could be divided into two groups, i.e., the first group included those from Nongkhai and Nakornphanom provinces with their genetic distant values of 0.20-0.36 and the second group involved those from Mukdaharn and Ubon Ratchatani provinces with their genetic distant values of 0.20-0.44. Although the results on similarity indices have split the fish into two groups yet the gap between lowest and highest correlation coefficient values were relatively small. The small differences found could perhaps attributable to the environmental conditions rather than selection pressure (Campana *et al*., 2000).

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