BIOTROPIA Vol. 19 No. 1, 2012: 42 - 50

MOLECULAR IDENTIFICATION AND PHYLOGENETIC RELATIONSHIP AMONG LOCAL, SANGKURIANG, AND AFRICAN CATFISH BASED ON RAPD MARKER

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Received 31 May 2011/Accepted 08 May 2012

ABSTRACT

In 2004, BBPBAT Sukabumi had introduced a new strain of catfish, namely Sangkuriang catfish. However, the Sangkuriang catfish showed different phenotype from their parental, that is African catfish. Therefore, taxonomic analysis is needed as to get a clearer picture about their taxonomic status and relationships with local and African catfish. Taxonomic study could be done using molecular markers as a taxonomic character. One of the molecular markers is *randomly amplified polymorphic DNA* (RAPD) marker. Using RAPD, species status is defined based on the presence and absence of specific band on each catfish. Phylogenetics relationships was inferred from phylogenetic tree which was built using UPGMA tool as implemented in NTSYS software based on the similarity of RAPD band pattern. The result showed that Sangkuriang and local catfish had specific RAPD markers, while African catfish had not. This means that all RAPD markers of African catfish were shared with those of two other catfish. Local catfish was distantly related to African and Sangkuriang catfish. Only one African catfish (D4) was genetically related to Sangkuriang catfish, while the rest of African catfish samples were genetically related to green catfish. This means that African catfish showed a very divergence genetic constituent.

Key words: *Clarias* sp., molecular identification, phylogenetic relationships, RAPD

INTRODUCTION

Indonesia's fish farmer commercially cultivates catfish species. Formerly, only one species was cultivated, namely local catfish *Clarias batrachus*. However, since 1985 local catfish has been replaced by newcomer species from Africa, namely African catfish (*C. gariepinus*). This is due to several advantages of African catfish compared to local catfish. These advantages include the followings : (1) it can be cultivated in pond with limited water and high seed number; (2) lower production cost, easier cultivation technology and; (3) higher resistance and growth rate (Siregar *et al.* 1993).

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However, inbreeding has led to reduce African catfish seed quality. This fact was proved by lower growth rate and reproduction (Sunarma 2004). Nurhidayat (2000) reported that there was an increasing asymmetry of body part and abnormality on African catfish. Moreover, Rustija (1999) has also proven the reduction of growth rate on African catfish.

Those facts have led the Centre for Freshwater Aquaculture (Balai Besar Perikanan Budidaya Air Tawar/BBPBAT) Sukabumi made some efforts to improve the quality of African catfish. Therefore, since 2000 BBPBAT Sukabumi has done some backcrossing efforts between African catfish parental and their offsprings. The result is that in 2004 a new strain of catfish was launched , namely Sangkuriang catfish based on the decree of Ministry of Marine and Fisheries Affairs No. 26/MEN/2004 (Sunarma 2004).

Sangkuriang catfish resulted from backcross between F2 female and F6 male of African catfish. F2 female used in those backcross was direct offspring of African catfish, which was introduced to Indonesia in 1985. According to previous observation this Sangkuriang strain showed different phenotype to their parental with higher growth performance and fecundity and also better food conversion than their African parental (Sunarma 2004). However, not much is known whether their phenotype alteration is followed by the change of their genetic constituent. Therefore, it is important to do an analysis of their taxonomic status using molecular characters to infer their relationships with local and African catfish.

There are two methods for relationships analysis, i.e. phenetics and phylogenetics analyses. Phenetic analysis can only be inferred from the similarities level among taxa without considering their evolutions, while phylogenetic relationships is inferred from similarities and differences among taxa considering their evolutionary history. Ackerman *et al.* (1988) noted that relationships among separated populations can be inferred using nucleic acid analysis such as DNA.

One of molecular markers which can be used in such study is randomly amplified polymorphic DNA (RAPD) markers. The main reason for RAPD utilization is that RAPD markers showed species specific characters. Other reasons are this technique only needs small amount of DNA template and no cloning, sequencing, and other molecular charaterizations are needed (Hadrys *et al.* 1992).

Allele variations on RAPD markers are shown by the presence and absence of amplified band on gel electrophoresis after stained in 1% of ethidium bromide solution. Amplification of RAPD markers was performed using one arbitrary primer. This amplification can result in some polymorphic genetic segments on one population, whereas other segments are monomorphic either in one or among populations (Hadrys *et al.* 1992). Level of RAPD variability suggests that RAPD technique is a useful method to answer several problems, including individual identification, parental analysis, strain identification, and phylogenetic analysis (Parker *et al.* 1998).

The potential of RAPD markers on several studies has been reported such as on population genetic studies and species identification. RAPD technique is a good technique for species identification such as for fish (Bardakci & Skibinski 1994; Bercsényi *et al.* 1998; Prioli *et al.* 2002; Jug *et al.* 2004), clams (Rego *et al.* 2002,

Klinbunga *et al.* 2004), and insects (Armstrong & Ball 2005). Moreover, RAPD markers had shown a high polymorphisms intraspecific levels, like in oysters (Hirschfeld *et al.* 1999; Klinbunga *et al.* 2000; Klinbunga *et al.* 2001), mussels (Rego *et al.* 2002), scallop (Patwary *et al.* 1994), abalone (Huang *et al.* 2000), and *Abra tenuis* (Holmes *et al.* 2004).

Information on genetic differentiation among species proved as useful for species identification in *Clarias* (Teugels *et al.* 1992; Agne`se *et al.* 1997; Rognon *et al.* 1998). However, similar studies on Asian catfish were still rare (Daud *et al.*, 1989; Na-Nakorn *et al.* 1998, 1999), specifically for Indonesian catfish. Therefore, it is important to do a research on molecular identification and phylogenetic relationships on Indonesian catfish, especially on local, African, and Sangkuriang catfish.

This research was aimed at knowing about (1) specific genetic characters of local, African, and Sangkuriang catfish based on RAPD markers; (2) genetic distances among local, African, and Sangkuriang catfish; and (3) phylogenetic relationships among local, African, and Sangkuriang catfish.

MATERIALS AND METHODS

This research used survey method by applying purposive random sampling. Local and African catfish were collected from Banyumas Regency, while Sangkuriang catfish was collected from BBPBAT Sukabumi, green catfish (*Hemibagrus nemurus*) was bought from a fisherman in Klawing River Purbalingga. DNA analysis was conducted at the Animal Taxonomy Laboratory, Faculty of Biology, Jenderal Soedirman University, Purwokerto.

Tissue samples were obtained from cut off of caudal fin of catfish and green catfish with the help of scissors and pinset. Fin clips were preserved in 96 % ethanol and stored at room temperature until DNA analysis.

Total genomic DNA was isolated using chelex methods (Walsh *et al.* 1994). RAPD markers amplified using ten arbitrary primers as follow: OPA-07 GAAACGGGTG, OPA-09 GGGTAACGCC, OPA-11 CAATCGCCGT, OPA-20 GTTGCGATCC, OPAC-14 GTCGGTTGTC, OPAH-01 TCCGCAACCA, OPAH-02 CACTTCCGCT, OPAH-04 CTCCCCAGAC, OPAH-08 TTCCCGTGCC, and OPAH-09 AGAACCGAGG (Muneer *et al.* 2009).

Amplification of RAPD was conducted in a total volume of 25 µl. PCR mixtures consisted of 16. 35 µl of H_2O, 2.5 µl 10X PCR buffer, 2 µl of $MgCl_2$, 1.5 µl dNTPs, 1 µl primer, 0.15 µl taq polymerase, and 1.5 µl template DNA. PCR mixtures were predenaturated for 3 minutes at 94°C and followed by 37 cycles as follows: denaturation at 94°C for 45 seconds, annealing for 30 seconds at 40°C and extension at 72°C for 1 minute. Final extension was performed at 72°C for 5 minutes (modified from Muneer *et al.* 2009). PCR product was migrated on 1% agarose gel electrophoresis and stained in 1% ethidium bromide solution. Stained gel was visualized under UV light transiluminator and photographed. Amplified PCR products (RAPD markers) were used for further analysis.

Specific bands were analyzed descriptively based on the presence or absence of bands for each catfish species. Phylogenetic relationships were started by transformation of qualitative to quantitative binary data (0:1). Phylogenetic tree was constructed using UPGMA cluster analysis as implemented in Numerical Taxonomy and Multivariate System (NTSYS) software version 2.0.

RESULTS AND DISCUSSIONS

Five individuals of each catfish species were selected for DNA isolation. Migration of isolated DNA on 1% agarose gel electrophoresis and stained gel on 1% ethidium bromide showed that smear DNA was obtained. Smear products are commonly obtained from samples preserved in ethanol and isolated using chelex methods. Another factor could be due to DNA fragmentation caused by physical treatment (Pharmawati 2009). However, these isolations products had enough quality as PCR templates, as shown by Kochzius & Nuryanto (2008) *Tridacna crocea*. Both authors obtained very good PCR product from DNA template isolated using Chelex methods (Fig. 1A). Clear PCR products were also resulted when COI DNA fragment of Tridacna maxima were amplified using Chwlwx DNA template (Nuryanto & Kochzius 2009, Fig. 1A). In addition, Nuryanto and Susanto (2010) also showed good RAPD marker from *P. erosa* when the total DNA were isolated using Chelex methods (Fig. 1B).

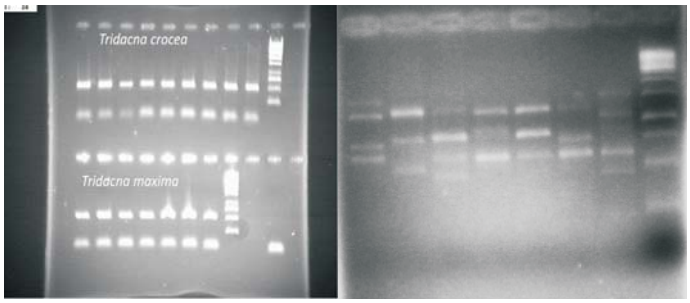

Figure 1. PCR product resulted from the amplification of Chelex template DNA.
Remarks: A : PCR product from *Tridacna crocea* and *T. maxima*
B : RPAD markers from *Polymesoda erosa*

PCR-RAPD markers were amplified using ten arbitrary primers. However, only six out of the ten primers could amplify RAPD markers from all catfish species including the out group species *Hemibagrus nemurus* (Bagriidae). Meanwhile, the rest four primers could not amplify RAPD markers from all catfish individuals. Those primers were OPAH_01, OPAH_02, OPAH_04, and OPAC_14. There are several factors for unsuccessful PCR reaction. These are including no complementary sequences between DNA template and primers, unspecific primer, and PCR conditions. Unspecific primer might cause either amplification of non target sequences or none amplified product (Rybicky 1996). Another possible factor of unsuccessful PCR

reactions could be due to the quality of DNA template resulted from Chelex methods was not good enough because of fragmentation. Fragmented DNA could reduce or even lost complemetary sequences between DNA template and the primer. The lost of complementary sequences between DNA template and the primer means that no PCR products will be produced during PCR reactions.

Six out of the ten primers were selected for RAPD markers amplification from catfish, whereas none of them could amplify RAPD markers from green catfish although several optimizations were performed. The six primers were OPA_07, OPA_09, OPA_11, OPA_20, OPAH_08, and OPAH_09. However, each primer had different capability to amplify RAPD markers. Primer OPA_07 resulted to seven RAPD markers ranges from 400 bp up to 1250 bp (Table 1). Primer OPA_09 amplified six PCR products. The length of the products were between 350 bp and 1000 bp (Table 1). Primer OPA_11 was only resulting one RAPD marker with 500 bp in length (Table 1). Meanwhile, primer OPA_20 could amplify seven RAPD markers with length ranges from 500 bp - 1400 bp (Table 1).

Amplification using primer OPAH_08 resulted to four RAPD markers (Table 1). The sizes of the markers were ranged between 500 and 1100 bp. Primer OPAH_09 was able to amplify six RAPD markers. The size of those RAPD markers ranges from 500 bp up to 1100 bp. High number of the amplified RAPD markers using six primers was due to those primers having complement nucleotide sequences to several sites on genomic DNA of studied catfish. Success of RAPD markers amplification depended on complementary primer to template DNA and PCR. Unspecific primer might led to miss priming and result to wrong target amplicon or no PCR product at all (Rybicky 1996). DNA fragments of PCR amplification product from each catfish are presented on Table 1.

Observation on resulted DNA bands pattern proved that each primer produced different DNA bands. The number of resulted DNA bands was mostly depending on how the primer recognized homologous sequences on template DNA. Higher number of attachment sites on DNA template with more number of DNA bands was obtained (Tingey *et al.* 1994). For primer development, composition, size, and their homology to template DNA has to be defined to obtain products during PCR reaction (Jamil 2005).

Table 1 shows that each catfish has specific and shared RAPD markers. Specific RAPD markers which differentiate between local catfish and all others catfish were OPA_09-350, OPA_20-600, OPA_20-500, OPAH_08-500, OPAH_09-1200, OPAH_09-750, and OPAH_09-500. Specific markers for Sangkuriang catfish were OPA_07-1250, OPA_09-600, OPA_20-1400, and OPAH_09-1500. Meanwhile, African catfish has not specific RAPD markers. All African catfish RAPD markers were shared either with local, Sangkuriang or both catfish.

The presence of specific markers on Sangkuriang catfish made it different from African catfish as their parental. However, those differences are related to their phenotypic differentiation such as growth rate and resistances to disease among them still need to be clarified.

Phylogenetic relationships among catfish are shown on Figure 2. The phylogenetic tree shows that all individuals of local catfish are grouped together in one clade

Table 1. RAPD markers amplified using six selected primers.

No	Marker	Local Catfish					African Catfish					Sangkuriang Catfish					Green Catfish				
		1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
1	OPA_07-1250	0	0	0	0	0	0	0	0	0	0	1	1	0	1	1	0	0	0	0	0
2	OPA_07-1000	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
3	OPA_07-900	1	1	1	1	1	0	0	1	1	0	1	1	1	1	1	0	0	0	0	0
4	OPA_07-750	1	1	1	0	1	0	0	1	1	0	1	1	1	1	1	0	0	0	0	0
5	OPA_07-600	1	1	1	1	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0
6	OPA_07-500	0	0	0	1	0	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0
7	OPA_07-400	1	1	1	0	1	0	0	0	0	0	1	1	1	1	1	0	0	0	0	0
8	OPA_09-1000	1	1	1	1	1	0	0	0	1	0	1	1	1	1	1	0	0	0	0	0
9	OPA_09-750	1	1	1	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
10	OPA_09-600	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0
11	OPA_09-500	1	1	1	0	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0
12	OPA_09-400	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0
13	OPA_09-350	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
14	OPA_11-500	1	1	1	1	0	0	0	0	1	0	1	1	1	1	0	0	0	0	0	0
15	OPA_20-1400	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
16	OPA_20-1100	0	0	0	1	0	0	0	0	1	0	1	1	1	0	0	0	0	0	0	0
17	OPA_20-1000	1	1	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
18	OPA_20-900	0	0	0	0	0	0	0	0	1	0	1	0	1	1	1	0	0	0	0	0
19	OPA_20-750	1	1	1	1	1	0	0	0	1	0	1	0	1	1	1	0	0	0	0	0
20	OPA_20-600	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
21	OPA_20-500	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
22	OPAH_08-1100	0	0	0	1	0	0	0	0	1	0	1	1	1	0	1	0	0	0	0	0
23	OPAH_08-900	1	1	1	1	1	0	0	0	1	0	1	1	1	0	0	0	0	0	0	0
24	OPAH_08-750	1	1	1	0	1	0	0	0	1	0	1	1	1	0	0	0	0	0	0	0
25	OPAH_08-500	1	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
26	OPAH_09-1600	0	0	0	0	0	0	0	1	1	0	1	1	1	0	1	0	0	0	0	0
27	OPAH_09-1500	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
28	OPAH_09-1200	1	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
29	OPAH_09-750	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
30	OPAH_09-600	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	0	0	0	0
31	OPAH_09-500	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

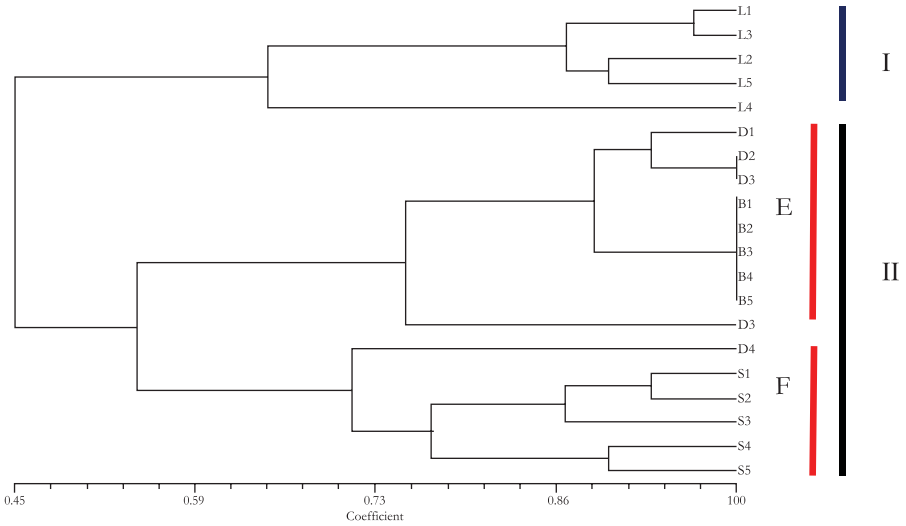

Figure 2. Phylogenetic tree showing the relationships among catfish

Remarks: I = clade I
II = clade II
E = subclade E
F = subclade F
L_{1-5} = local catfish
D_{1-5} = African catfish
S_{1-5} = sangkuriang catfish
B_{1-5} = green catfish

(clade I). African, Sangkuriang, and green catfish made another clade (clade II). Moreover, clade II could be divided into two subclades i.e. subclade E and F. Subclade E consists of four individuals of African catfish (D_1, D_2, D_5, D_3) and all individuals of green catfish (B_{1-5}), whereas subclade F is made from one individual of African catfish (D4) and all individuals of Sangkuriang catfish (S_{1-5}). The tree could also explain that African catfish showed a very high genetic divergence because individuals of African catfish did not occur on one clade. Four individuals belong to subclade E and one individual to subclade F (D4) Moreover, if we come into detail, although D1, D2, D5 and D3 were placed in one subclade, however only D2 and D5 had similar genetic constituent, while D1 was separated from D2 and D5 by about of 0.09 coeficient divergence (Figure 2). D3 was separated from D1, D2, and D5 by about of 0,24 coefficient divergence (Figure 2). This could be proven by subclade E which made from African and green catfish. Subclade F has proven that Sangkuriang catfish are the offspring of African catfish since all individuals of Sangkuriang are descendant of D4 African catfish. Although only one individual of African catfish was the parental of Sangkuriang catfish, this research supported the statement from Sunarma (2004) that Sangkuriang catfish resulted from backcross between F2 female and F6 male of African catfish.

Another interesting thing was that four individuals of African catfish were grouped together with green catfish. This phenomena could be due to that the used marker (RAPD) was not relliable enough to differentiate African catfish and green catfish. Therefore, to separate both species, powerful markers are needed such as perhaps DNA sequences and microsatellite markers. Other possibility would be due to the fragmented DNA template which lead to the lost of some complementary sequences between primer and DNA template on both species and resulted to similar RAPD pattern (this does not explain why the other 4 African catfish are in clade A).

CONCLUSIONS

Based on result and discussion it could be concluded that local and Sangkuriang catfish had specific RAPD bands, while African catfish did not. African catfish was phylogenetically closely related to Sangkuriang local catfish which has high genetic distance to African and Sangkuriang catfish. Small fraction (D4) of African catfish already proved that they are the parental of Sangkuriang strain. African catfish showed high genetic divergence, as shown by loss of marker.

REFERENCES

Ackerman E, Ellis LBM, Williams LE. 1988. Biophysics. Redjani and Abdulbasir (translators). Airlangga University Press, Surabaya.

Agne`se JF, Teugel GG, Galbusera P, Guyomard R, Volckaert F. 1997. Morphometric and genetic characterization of sympatric populations of *Clarias gariepinus* and *C. anguillaris* from Senegal. J Fish Biol 50: 1143–57.

Armstrong KF, Ball SL. 2005. DNA barcodes for biosecurity: invasive species identification. Phil Trans R Soc B 360: 1813–23.

Bardakci F, Skibinski DOF. 1994. Application of the RAPD technique in tilapia fish: species and subspecies identification. Heredity 73: 117-23.

Bercsényi M, Magyary I, Urbányi B, Orbán L, Horváth L. 1998. Hatching out goldfish from common carp eggs: interspecific androgenesis between two cyprinid species. Genome 41: 573-579.

Daud DK, Patimah I, Kijima A. 1989. Genetic variability and relationships among four species of freshwater catfish. Malays Appl Biol 18: 23–31.

Hadrys H, Balick M, Schierwater B. 1992. Applications of random amplified polymorphic DNA (RAPD) in molecular ecology. Molecular Ecology 1: 55–63.

Hirschfeld BM, Dhar AK, Rask K, Alcivar-Warren A. 1999. Genetic diversity in the eastern oyster (*Crassostrea virginica*) from Massachusetts using the RAPD technique. J Shellfish Res 18: 121–25.

Holmes SP, Dekker R, Williams ID. 2004. Population dynamics and genetic differentiation in the bivalve mollusc *Abra tenuis*: aplanic dispersal. Mar Ecol Prog Ser 268: 131–40.

Huang BX, Peakall R, Hanna PJ. 2000. Analysis of genetic structure of blacklip abalone (*Haliotis rubra*) populations using RAPD, minisatellite and microsatellite markers. Mar Biol 136: 207–16.

Jamil I. 2005. Analysis of ribosomal DNA ITS sequences (rDNA) and primer design for *Phytophthora palmivora* Butl. detection on Cacao. Thesis. Graduate School. Bogor Agricultural University Bogor, Bogor. 48 pp.

Jug T, Dovc P, Pohar J, Snoj A. 2004. RAPD analysis as a tool for discriminating marble trout from hybrids (marble trout · brown trout) in the zones of hybridization. J Anim Breed Genet 121: 156-62.

Klinbunga S, Amparyup P, Leelatanawit R, Tassanakajon A, Hirono I, Aoki T, Jarayabhand P, Menasveta P. 2004. Species Identification of the Tropical Abalone (*Haliotis asinina*, *Haliotis ovina*, and *Haliotis varia*) in Thailand Using RAPD and SCAR Markers. J Biochem Mol Biol 37 (2): 213-22.

Klinbunga S, Ampayup P, Tassanakajon A, Jarayabhand P, Yoosukh W. 2000. Development of species-specific markers of the tropical oyster (*Crassostrea belcheri*) in Thailand. Mar Biol 2: 476–84.

Klinbunga S, Ampayup P, Tassanakajon A, Jarayabhand P, Yoosukh W. 2001. Genetic diversity and molecular markers of cupped oysters (genera *Crassostrea*, *Caccostrea*, and *Striostrea*) in Thailand revealed by randomly amplified polymorphic DNA analysis. Mar Biotechnol 3: 133–144.

Kochzius M, Nuryanto A. 2008. Strong genetic population structure in the boring giant clam *Tridacna crocea* across the Indo-Malay Archipelago: implications related to evolutionary processes and connectivity. Mol Ecol 17: 3775-87.

Muneer PMA, Gopalakrishnan A, Musammilu KK, Mohindra V, Lal KK, Basheer VS, Lakra WS. 2009. Genetic variation and population structure of endemic yellow catfish, *Horabagrus brachysoma* (Bagridae) among three populations of Western Ghat region using RAPD and microsatellite markers. Mol Biol Rep 36:1779–91.

Na-Nakorn U, Taniguchi N, Nugroho E, Seki S, Kamonrat W. 1999. Isolation and characterization of microsatellite loci of *Clarias macrocephalus* and their application to genetic diversity study. Fisheries Sci 65: 520–26.

Na-Nakorn U, Hara M, Taniguchi N, Seki S. 1998. Isozyme variation of *Clarias macrocephalus* from four locations in Thailand. Fisheries Sci 64: 526–30.

Nurhidayat MA. 2000. Asymmetry fluctuation and abnormality of African catfish (*Clarias* sp.) from tree region in Java Island. Thesis. Graduate School. Bogor Agricultural University Bogor, Bogor.

Nuryanto A, Susanto AH. 2010. Genetic variability of *Polymesoda erosa* population in the Segara Anakan Cilacap. Biotropia 17(1): 22 – 30.

Nuryanto A, Kochzius M. 2009. Highly restricted gene flow and deep evolutionary lineages in the giant clam Tridacna maxima. Coral Reefs 28:607–19.

Parker PG, Snow AA, Schug MD, Booton GC, Fuerst PA. 1998. What molecules can tell us about populations: choosing and using a molecular marker. Ecology 79 (2): 361–82.

Pharmawati M. 2009. Optimization of DNA extraction and PCR-RAPD on *Gravellea* spp. (Proteaceace). Jurnal Biologi 8(1): 12 – 6.

Patwary MU, Kenchington EL, Bird CJ, Zouros E. 1994. The use of random amplified polymorphic DNA markers in genetic studies of the sea scallop, *Placopecten magellanicus* (Gmelin, 1791). J Shellfish Res 13: 547–53.

Prioli SMAP, Prioli AJ, Júlio Jr. HF, Pavanelli CS, de Oliveira AV, Carrer H, Carraro DM, Prioli LM. 2002. Identification of *Astyanax altiparanae* (Teleostei, Characidae) in the Iguaçu River, Brazil, based on mitochondrial DNA and RAPD markers. Genet Mol Biol 25 (4): 421-30.

Rego I, Martianez AS, Lez-Tizoa NAG, Vieites J, Leira F, Meandez J. 2002. PCR Technique for Identification of Mussel Species. J Agric Food Chem 50: 1780-84.

Rybicky EP. 1996. PCR primer design and reaction optimisation. In Molecular Biology Techniques Manual. Ed. V.E. Coyne, M.D. James, S.J. Reid & E.P. Rybicki. Dept.of Microbiology. Univ. Cape Town.

Rognon X, Teugels GG, Guyomard R, Galbusera P, Andriamanga M, Volckaert F, Agne`se JF. 1998. Morphometric and allozyme variation in the African catfishes *Clarias gariepinus* and *C. anguillaris*. *J Fish Biol* **53:** 192–207.

Rustija. 1999. Genetic quality improvement of African catfish and cryopreservation. Paper presented at Pertemuan Perekayasaan Teknologi Pembenihan Agribisnis Ikan Air Tawar, Payau dan Laut. Direktorat Jenderal Perikanan. Departemen Pertanian, Jakarta.

Siregar S, Putra RM, Sukendi. 1993. Fish fauna at Sector Bukit Tigapuluh waters in Siberida, Sumatra. Rain Forest and Resource Management. Proceedings of the NORINDA. Jakarta, 23-25 Mei 1993.

Sunarma A. 2004. Improvement of Sangkuriang (*Clarias* sp.) productivity. Balai Budiaya Air Tawar, Sukabumi

Teugels GG, Guyomard R, Legendre M. 1992. Enzymatic variation in African clariid catfishes. J Fish Biol 40: 87–96.