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Genetic Kinship of Nilem Fish Strains (*Osteochillus hasselti)*

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Authors' contributions

This work was carried out in collaboration among all authors. Author YA designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors IDB and US managed the analyses of the study. Author AM managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

The research aims to analyze the kinship of nilem fish strains using RAPD (Random Amplified Polymorphic DNA). This research used exploratory method with descriptive analysis. The primers used in the RAPD method are OPA-02 (5'-TGCCGAGCTG-3 '), OPA-03 (5'-AGTCAGCCAC-'3), OPA-05 (5'-AGGGGTCTTG-3') and OPA-11 (5) 5'- CAATCGCCGT-3 '). The primary optimization results showed that OPA-02 and OPA-11 were the primary primers for detecting polymorphic and monomorphic fragments of the nilem fish genome. Phenograms from the RAPD method that were processed through the NTSYS-pc program showed that the OPA-02 primer was the best primer for nilem kinship analysis. Relationship between red nilem with green nilem is 92%, red nilem and green nilem with mangot nilem are 72% and the three nilem with *beureum panon* are 12%.

Keywords: Kinship; RAPD; nilem strain.

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1. INTRODUCTION

Nilem fish is an endemic fish in West Java that has good prospects to be farmed because its eggs can be used as export commodities and baby fish products for domestic market needs. However, industrial-scale of nilem cultivation carried out through artificial spawning can result in the decrease of reproduction capabilities of parent fish in the form of decreased fecundity, egg quality and decreased ability of nilem fish to adapt to the environment.

Development of nilem aquaculture needs to be done to increase the production of nilem and currently there are four strains developed in the Priangan area (Sumedang, Garut, Singaparna, Tasikmalaya) local namely red nilem, green nilem, mangot nilem and *bereum panon* (Fig. 1).

Nilem is a mass spawner (synchronous batch spawner) and egg fecundity that varies between the existing nilem strains [1]. This variation is a reflection of polymorphism that will be shown in the differences in phenotype and genotype in the genome of the nilem strain.

Selective hatchery programs in nilem are rarely carried out. Generally, hatchery programs favor the crossing of different strains through cross breeding [2]. Artificial breeding is very helpful in increasing the value of nilem fish seed production, but cross breeding is likely to have a negative impact if the broodstock used in fact has a close kinship [3].

The selection program can be implemented if the genetic diversity of the nilem fish is high, and if the genetic diversity is low (kinship between close strains) it may be possible to cross (hybridize) [4]. Information on genetic variation based on genotypic data obtained from polymorphic fragments resulting from amplification of the genome of the nilem fish is useful to determine the close proximity of genetic kinship between strains. Based on the test results using the RAPD-PCR (Random Amplified Polymorphic DNA-Polymerase Chain Reaction) method, a phylogram (phenogram) will be obtained based on genetic data that is in accordance with the morphological characteristics (phenotype) of Nilem fish strains. Furthermore, the kinship data of this nilem fish strain can be used in designing a superior nilem fish breeding program.

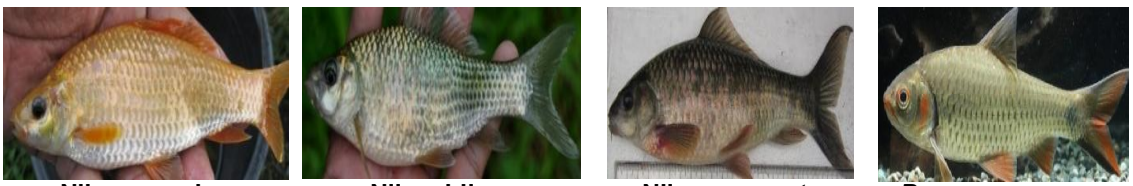
2. RESEARCH MATERIALS AND METHODS

The research method used was exploratory method without using experimental design. Data obtained from research in the form of polymorphic and monomorphic DNA fragments resulting from amplification of the RAPD-PCR method were further analysed using the NTSYS (Numerical Taxonomy and Multivariate Analysis System) program to produce phylogenic trees that declared kinship among the four nilem fish strains.

The materials used in the study include nilem as research objects, chemicals, a set of DNA amplification and electrophoresis.

Fish samples: Fish samples used in this study were red nilem, green nilem, mangot nilem and panon beurem. The nilem strain was obtained from the Ciherang Public Aquatic Fisheries Conservation Center and Ciparanje outdoor hatchery, Faculty of Fisheries and Marine Sciences, Universitas Padjadjaran. Genomic DNA samples from each strain were taken from a part of the brain as a template DNA source.

DNA Extraction: The sample used was a part of the fish's brain and then the sample was put into a plastic bottle that had been added preservation solution with a composition of 96% alcohol and glycerol (4: 1) for DNA extraction.



Nilem merah **Nilem hijau** **Nilem mangot** ***Beureum panon***

Fig. 1. Nilem fish strain (personal documentation)

DNA extraction was carried out by using the wizard® genomic purification kit (Promega, Madison, WI, USA). Brain samples were weighed and 1.5 ml and put into eppendorf tubes then crushed using plastic chopsticks and 300 ml of nucleic lysis solution was added. Then the sample was incubated in a water bath at 65ºC for 15 minutes. After that, 1.5 µl RNase solution was added to the sample and then homogenized and then incubated again at 37ºC for 20 minutes and dried. The next step is to add 100 µl of protein precipitation solution to the sample then the sample was homogenized and cooled on ice for 5 minutes. The solution was then centrifuged for 4 minutes at 13,000 rpm. After a precipitate has formed at the base of the microtube, then the supernatant was taken at the top of the tube and transferred to a new eppendorf tube, and 300 µl of isopropanol were added at room temperature. Subsequently, the solution was centrifuged again for 1 minute at a speed of 13,000 rpm. The supernatant at the top of the tube is removed, then mixed with 300 µl of 70% ethanol at room temperature, then centrifuged again for 1 minute at a speed of 13,000 rpm. Ethanol was removed, and the DNA pellet bound to the bottom of the tube was dried for 15 minutes by turning the Eppendorf tube upside down over a dry tissue. The pellet is then dried by adding 50 µl rehydration solution, then incubated for 1 hour in a water bath at 65ºC, the resulting solution is a DNA sample solution.

The quantity and quality of the DNA sample as the primary template used needs to be measured to determine the concentration and purity of the sample. The formula for calculating DNA concentration [5] is as follows:

DNA concentration (ng / µl) = 50 ng / µl x OD260 x dilution factor

Dilution factor = 50 times

The ratio between the readings of DNA sample spectrophotometry at A260nm and A280nm determines the level of DNA purity. The quality of DNA isolated is said to be pure if the ratio of the two values ranges between 1.8-2.0 [6].

RAPD Primary Optimization: PCR optimization is needed considering the RAPD primary sequences stick randomly along the DNA of the sample fish genome so that only the primary sequences that are complementary to the DNA sequences of the genome produce DNA fragments. Considering that nilem species have taxonomic similarities, especially the order of Cypriniformes and Cyprinidae family with carp, RAPD primary selection refers to the results of studies on carp for primary use of RAPD of nilem fish.

Four primers of RAPD (OPA-2, OPA-3, OPA-5 and OPA-11) have been selected by referring to the results of Muharam (2010) and Mulyasari (2010) research to identify polymorphisms in koi fish (*Cyprinus carpio koi*) and nilem (*O. hasselti*). The results show that the suitable RAPD primers for nilem are OPA-2 and OPA-11 in base order as listed in Table 1. Thus these two primers are used as a reference for identification of kinship in nilem fish studies.

Table 1. RAPD primary optimization for nilem

Primer Name	Base Sequence
OPA– 02	TGCCGAGCTG
OPA– 11	CAATCGCCGT

DNA Amplification with RAPD-PCR Technique: Amplification of striped catfish genome DNA was carried out using the RAPD-PCR method to see a marker of polymorphism and kinship in fish species. This amplification uses two primary types of Operon Technology namely OPA-2 and OPA-11. Each sample was reacted with 12.5 µl 2X go taq® green master mix (Promega, Madison, WI, USA), 1.25 µl RAPD primers, 2 µl DNA samples as a template and 9.25 µl sterile Nuclease Free Water was added until the overall total reaction volume is 25 µl. PCR reaction was carried out using 45 thermal cycler replication cycles starting from denaturation, annealing and extension stages. Before the denaturation stage begins, the process started with the stage of pradenaturation at a temperature of 95ºC for 2 minutes (1 cycle). Each replication cycle consisted of denaturing at 95ºC for 1 minute, annealing at 36ºC for 1 minute and extension at 72ºC for 2 minutes. After 45 cycles, the process was ended with final extension at 72ºC for 5 minutes [7]. The amplification product was then electrophorized for 85 minutes at 75V in 1.4% agarose gel.

Kinship Strains: Analysis of genetic kinship of the nilem fish sample was done by inputting the presence or absence of DNA fragments from the amplification results that were translated into numerical data. The DNA amplicon that appears translates into number one (1) and what does not appear is translated to number zero (0). DNA fragments that are only amplified in a stripped

catfish sample and not amplified in another catfish sample are interpreted as polymorphic fragments that indicate genetic variation and differences in genetic distance between an organism [8].

The genetic relationship of the nilem DNA sample is determined by calculating its similarity index based on amplified numeric data. This safety index is calculated using the NTSYS program. Kinship among nilem fish was analyzed using genetic distance based on the modified Nature et al. UPGMA (Unweighted Pair Group Method with Arithmetic Averages) program. (2006) from the NTSYS software. Data generated from the use of the program in the form of phylogeny tree construction is presented in the form of a phenogram.

3. RESULT S AND DISCUSSION

3.1 DNA Extraction

DNA isolation or extraction carried out on test samples is intended to obtain DNA pellets that can be used as templates. This extraction process includes the process of tissue lysis, the process of separating and dissolving cell organelles with the cell nucleus, the process of settling extracted remnants, and washing DNA pellets from debris (residual extraction results) so that only DNA pellets are obtained [9]. The existence of genomic DNA isolation results of catfish sample can be demonstrated from the results of 1.4% agarose gel electrophoresis (Fig. 2). Based on the 1kb DNA marker, the size of the genome DNA is above 10000 bp (10 kb) because DNA consists of exons and introns so that the size of the DNA fragments of the genome of the nilem brain (Fig. 2) is longer than that of the 1 kb DNA marker.

The quality of genomic DNA as a template is indicated by measurements of DNA concentration and purity values obtained from spectrophotometric measurements that have been carried out based on the ratio of absorbance ratio values of A260 nm and A280 nm. The concentration of genomic DNA can be determined by calculating the value of A260 nm times 50 mg / ml multiplied by the dilution factor (50x) [5]. The results of DNA concentration and purity calculations are presented in Table 2.

The result of genomic DNA extraction which has a good purity level is between 1.8-2.0 [10]. The absorbance ratio at wavelengths of 260 and 280 (A260 / A280) which is above the range DNA purity values indicates that there is RNA contamination, while purity value below 1.8 indicates that there is protein contamination. Good genomic DNA concentration values are more than 50 µg / ml [5]. Based on the results obtained above, nilem fish brain genome DNA ranged from 125.0 to 672.5 µg / ml while its purity ranged from 1.667 to 1.975, so the genomic DNA was suitable to use in the amplification process.

3.2 Nilem Fish DNA Amplifiers

Nilem fish genome DNA amplification results using OPA-02 primers is presented in Fig. 3. DNA fragments produced vary because there are differences in the order of the nucleotide bases where the primary attachment is attached. The variation of the DNA band depends on the suitability of the template and primer used with different size of the amplicon pliers [11].

DNA fragments can be grouped into two categories, namely polymorphic bands and monomorphic bands. Polymorphic bands are images of DNA bands that appear in certain sizes, but in other DNA sample, bands are not found at that size [12]. Monomorphic band is a band found in most fish samples with the same fragment size so that it has no variation. DNA amplified by OPA-02 primers ranging from 1856 to 367 bp is shown in Table 3.

Based on Table 3, *beureum panon* fish (NBP2) has the most amplified bands, with 16 DNA bands. Whereas the red nilem (NM2) has 6 DNA bands, the green nilem (NH2) has 7 DNA bands and the mangot nilem (NMG2) has 4 DNA bands.

Table 2. The value of DNA purity of the genome of the nilem fish brain

No	Sample	DNA concentration (µg/ml)	DNA Purity		Purity
			Abs 260nm	Abs 280nm	
1	Nilem Merah	197,5	0,079	0,040	1,975
2	*Beureum panon*	145,0	0,058	0,034	1,706
3	Nilem Hijau	672,5	0,269	0,158	1,703
4	Nilem Mangot	125,0	0,050	0,030	1,667

Abs : absorbansi

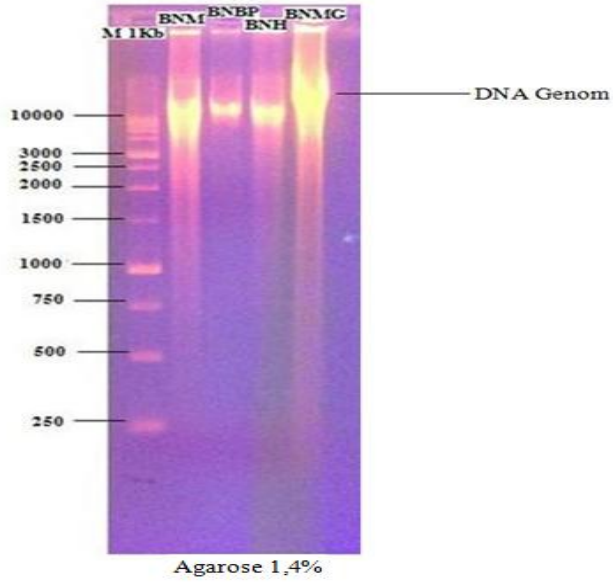


Fig. 2. Electrophoregram DNA of the brain genome of the red nilem, *beureum panon*, green and mangot

M 1Kb: Marker DNA ladder 1kb; BNM: Red Nilem Brain; BNBP: Brain Nilem Beureum panon; BNH: Green Nilem Brain; BNMG: Brain Nilem Mangot

Table 3. Polymorphic and monomorphic fragments of the nilem (OPA-02)

Fragment Distance (bp)	NM2	NBP2	NH2	NMG2
1856		--*		
1625		--*		
1467		--*		
1418		--*		
1341		--*		
1216		--*		
1155		--*		
1098		--*		
1056		--*		
1017	--		--	
933		--*		
757	--		--	--
726	--		--	--
698	--	--	--	--
647		--*		
599		--*		
555		--	--	
454	--		--	
433	--		--	--
413		--*		
367		--*		

-- : Monomorphic fragment; -- : Polymorphic fragment*

NM2: Red Nilem; NBP2: Nilem Beureum panon; NH2: Green nilem; NMG2: Nilem Mangot (primer OPA-02)

OPA-02 amplification polymorphic bands are found in the *beureum panon*, ranged in sizes 1856 bp, 1625 bp, 1467 bp, 1418 bp, 1341 bp, 1216 bp, 1098 bp, 1155 bp, 1056 bp, 933 bp, 647 bp, 599 bp , 413 bp, and 367 bp. The polymorphic band was not found in samples of other nilem strains. The polymorphic fragments of the *beureum panon* fish may be due to the morphological characteristics of the fish. The phenotype of *beureum panon* has specific

characteristics compared to other nilem fish, which has a silvery body color such as milkfish, with a relatively small body size and unique characteristic such as red spots on the operculum, and red eyes.

In the sample of red, green and mangot nilem there is no variation of DNA bands or polymorphic fragments but has different DNA fragments between the three nilem. In the green nilem there is a different band with the red nilem and mangot that is in fragments 555 bp (Table 3). These different DNA fragments are representations for different phenotypes between green nilem and red nilem. Overall polymorphic fragments are only found in *beureum panon* and not found in red, green and mangot nilem, indicating that the genetic distance of the *beureum panon* with the other three strains is far.

In addition to OPA-02 primers, OPA-11 primers also produce polymorphic bands in four nilem strains (Fig. 4).

Fragments amplified using OPA-11 primers (Fig. 4) have 3 DNA bands on the red nilem (NM11), 10 DNA bands on the *beureum panon* (NBP11), 4 bands on the green nilem (NH11) and 6 bands on the mangot nilem (NMG11) . The size of the visualized tape is in the range of 2462-268 bp. There are two strains that produce polymorphic fragments; *beureum panon* with 7 fragments and mangot nilem with 3 fragments (Table 4). Based on the number of polymorphic and monomorphic fragments (Table 4), *beureum panon* fish have the most DNA fragments of 10, whereas red nilem has 3, green nilem 4 and mangot nilem have 6 DNA fragments. The nilem *beureum panon* strain has as many as 7 polymorphic fragments amplified at 2462 bp, 1498 bp, 1156 bp, 986 bp, 885 bp, 356 bp and 298 bp. In contrast, in mangot strains there were three variations of DNA fragments amplified at sizes 1325 bp, 1190 bp, and 1092 bp.

This polymorphic fragment of the *beureum panon* strain can be represented by morphological characteristics of fish which is silvery color that is darker in the upper part of the body and brighter in the abdomen. The distinctive feature that distinguishes the *beureum panon* from the other nilem is the red spot on the operculum, red eyes, and a black line behind the operculum. The body size of the *beureum panon* is relatively small compared to red nilem, green nilem and mangot nilem [13].

Mangot strains also have phenotypic variations which are indicated by the presence of polymorphic fragments (Table 4). This phenotype variation of the nilem strain is related to its morphological characteristics, where the nilem mangot mouth which is located at the tip of the lower nose, pectoral fins, reddish ventral fins, and tail shape which resembles crescent moon that differentiates it from other nilem strains. OPA-11 primers can identify genetic similarities found in red and green Nilem that are indicated by monomorphic fragments at the fragment size position of 268 bp and 568 bp (Table 4). In green nilem, DNA fragments are different from red nilem, which is 338 bp in size. These different DNA fragments may be fragments that produce a silvery color on the green nilem which is similar to the *beureum panon*.

Table 4. Polymorphic and monomorphic fragments of nilem strain (OPA-11)

Fragment Distance (bp)	NM11	NBP11	NH11	NMG11
2462		--*		
1498		--*		
1444	--	--		
1325				--*
1190				--*
1156		--*		
1092				--*
986		--*		
885		--*		
568	--	--	--	
356		--*		
338		--	--	--
298		--*		
276			--	--
268	--		--	--

--: Monomorphic fragments; -- : Polymorphic fragments NM11: Red Nilem; NBP11: Nilem Beureum panon; NH11: Green Nilem; NMG11: Nilem Mangot (primer OPA-11)*

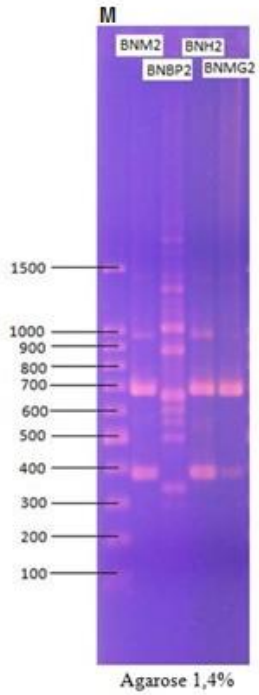


Fig. 3. Nilem genomic DNA amplificators (OPA-2)

M: Marker DNA ladder 1kb; BNM2: Brain Nilem Merah; BNBP2: Brain Nilem Beureum panon; BNH2: Brain Nilem Hijau; BNMG2: Brain Nilem Mangot

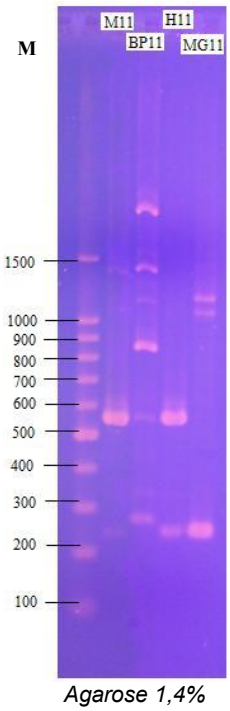


Fig. 4. Nilem genomic DNA amplicon with OPA-11 primers

M : Marker DNA ladder 1kb; M11: Red Nilem; BP11: Nilem Beureum panon; H11: Green Nilem; MG11: Mangot Nilem (primer OPA-11)

3.3 Phylogenetic Analysis of Nilem Strains

RAPD variations have been widely used to investigate the population structure of various types of fish [14] such as nilem fish [4], tilapia [15] and families Cichlidae, Mugilidae , Sparidae and Serranidae [16]. The existence of DNA bands that emerged from the amplification results using RAPD primers was translated into a binary matrix which was then used for simple matching equation analysis to determine the level of kinship between the samples of the nilem fish studied. According to [17], UPGMA (Unweighted Pair Group Method with Arithmetic Averages) analysis is the processing of binary matrix data into a kinship tree (phenogram) which shows the percentage of phenotypic similarity of each sample of the nilem strain. Phenograms obtained using OPA-2 and OPA-11 primers showed variable results (Figs. 5 and 6).

Kinogram of nilem fish samples using OPA-02 primers showed that the highest kinship index was found in red nilem samples with green nilem which was 92% then mangot nilem samples had a kinship index of 72% of red and green nilem. This shows that mangot nilem has a high level of kinship with red and green nilem.

Kinship index of *beureum panon* strain with the other three nilem strains is only 12%, this shows that the strain's genetic relationship is far away. Phenograms produced from OPA-11 primers differ from phenograms from OPA-02 (Fig. 6).

In the Nilem strain philogram using OPA-11 primers, green nilem samples and mangot nilem have a kinship index of 60%, it shows that the level of kinship between the two samples is still relatively high. Kinship index between mangot nilem samples with green nilem and red nilem is 35.25%, which means that the similarity level of red nilem strains with mangot nilem and green nilem is relatively low whereas for *beureum panon* samples the lowest kinship index is 24% with the other three strains. OPA-02 primer was able to produce monomorphic DNA fragments in each sample (indicated by the high genetic similarity index of red, green, mangot nilem strains) but polymorphic fragments were only found in the highest number of *beureum panon* (NBP2) samples. This indicates that OPA-02 primers work optimally on *beureum panon* (NMG2) samples. Instead the phenogram produced by OPA-11 polymorphic band primers showed low genetic similarity between red, green and mangot nilem strains. Based on the results of the two phenograms, OPA-02 primers provide the interpretation of genetic kinship of the nilem strain better than OPA-11.

Phenograms of the two primers show that both primers interpret a different kinship index from each sample. Based on the phenogram in Fig. 6 the similarity index of *beureum panon* with red nilem, green nilem and mangot nilem is 24%, which shows that *beureum panon* has a close kinship with the three nilem strains (primary OPA-11). *Beureum panon* strains have more similarities with the genus Tawes (Puntius) [18,13]) which are also shown in Fig. 5 with the highest number of polymorphic fragments (primary OPA-02).

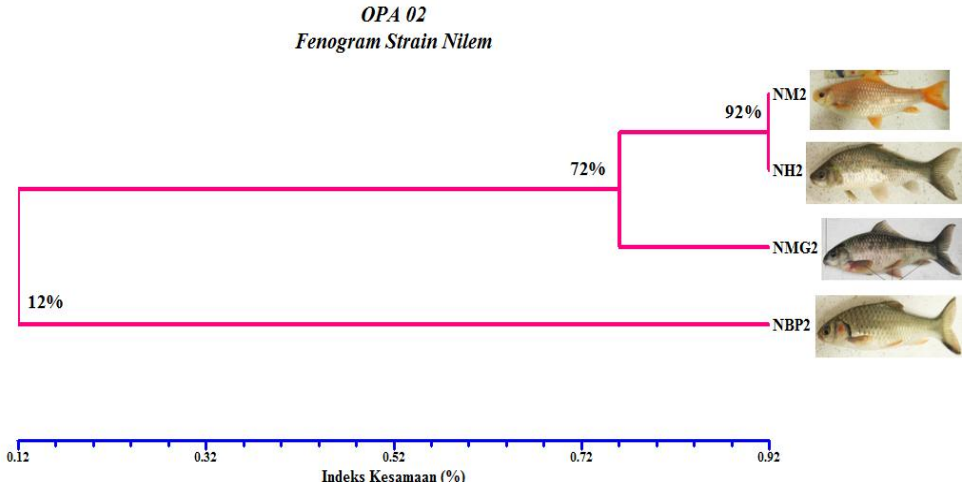


Fig. 5. Nilem strain phenogram using OPA-02 primers

NM2: Red Nilem; NH2: Green Nilem; NMG2: Nilem Mangot; NBP2: Nilem Beureum panon (primer OPA-02)

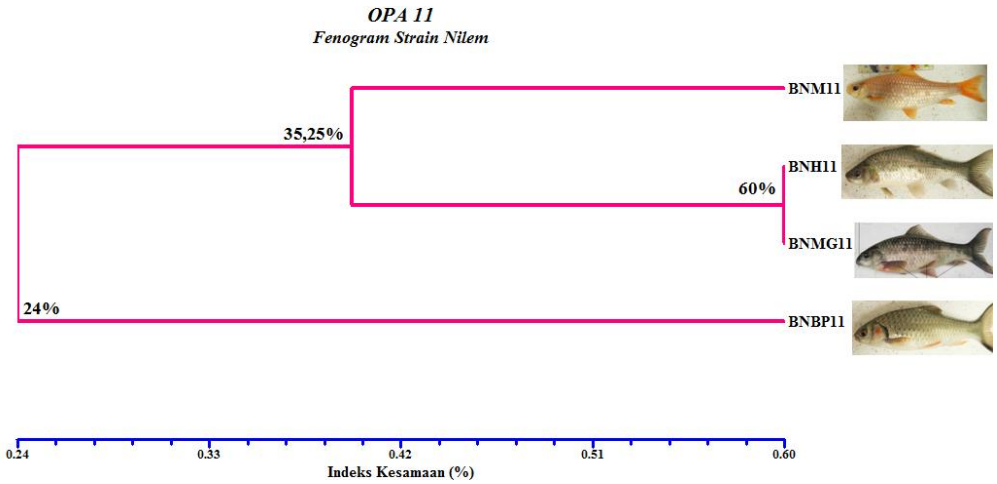


Fig. 6. Phenogram of the nilem strain using OPA-11 primers

BNM11: Red Nilem; BNH11: Green Nilem; BNMG11: Nilem Mangot; BNBP11: Nilem Beureum panon (primer OPA-11)

4. CONCLUSIONS

Based on the results and discussion, it can be concluded as follows:

1. OPA-02 primer is suitable for determining kinship among nilem strains.
2. Genetic distance of red and green nilem is 92%, and genetic distance of mangot nil is 72%, and genetic distance between the three nilem with *beureum panon* is 12%.

5. SUGGESTIONS

Suggestions that can be given for further research included:

1. Research on hybridization of green nilem with other strains is needed for the purpose of increasing egg production.
2. RAPD-PCR analysis is recommended to determine the potential parent of superior nilem breeds to increase egg production in nilem strains.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Prayogo NA, Wijayanti GE, Murwantoko Kawaichi M, Astuti P. Structure and phylogenetic of GnRH Genes of Hard-lipped Barb *(Osteochilus hasselti C.V).* Middle-East Journal of Scientific Research. 2011;10(3).
2. Nielsen HM, Odegard J, Olesen I, Gjerde B, Ardo L, Jeney G, Jeney Z. Genetic analysis of common carp (*Cyprinus carpio*) Strains I: Genetic parameters and heterosis for growth traits and survival. Aquaculture. 2010;303(10):14–21.
3. Jewel MAS, Rahman MN, Islam MN. Study of genetic variation in different hatchery Populations of Common Carp (*Cyprinus carpio*) of Mymensingh District in Bangladesh Using Microsatellite DNA Markers. J.Bio-sci. 2006;14:113–120.
4. Mulyasari. Karakteristik Fenotipe Morfomeristik dan Keragaman Genotipe RAPD (*Randomly Amplified Polymorphisme DNA*) Ikan Nilem (*Osteochilus hasselti*) di Jawa Barat. Skripsi FPIK IPB. Bogor; 2010.
5. Barbas CF, Burton DR, Scott JK, Silverman GJ. Quantitation of DNA and RNA. Cold Spring Harbor Laboratory Press. Cold Spring Harbor. NY, USA; 2001.
6. Sambrook J, Russel DW. Molecular Cloning: A Laboratory Manual. New York: Cold-Spring Harbor Laboratory Press. 2nd edition. 2001;165.
7. Muharam EG. Analisis Kekerabatan Ikan Mas Koi (*Cyrprinus Carprio Koi*) Dan Ikan Mas Majalaya (*Cyrprinus Carprio*) Menggunakan Metode Rapd. Skripsi. Universitas Padjadjaran. Jatinangor; 2010.
8. Liu ZJ, Cordes JF. DNA marker technologies and their applications in

aquaculture genetics. Aquaculture. 2004; 238:1-37.
9. Sano N, Morkwake M, Hondo R, Sano T. Herpes virus cyprini: A search for viral genome in infected fish. J. Fish Dish. 2005;16:495 –499.
10. Prayuni K. Isolasi dan Pengklonan Promoter Gen *lea3* yang Terinduksi Kekeringan Dari Tanaman Padi (*Oryza sativa* L.) Lokak Indonesia Kultivar Rojolele dan Batutegi. Skripsi. FMIPA Universitas Indonesia; 2008.
11. Ferguson AJ, Taggart AJ, Prodohl PA, Hynes RA. The application of molecular markers to study and conservation of fish populations with special refference to salmo. Journal of Fish Biology. 1995;47: 103-126.
12. Shivashankar M.. Random Amplified Polymorphic DNA (RAPD) Markersin Anticancer Drug Plants. International Journal of Current Microbiology and Applied Sciences. 2014;3(7):1091-1101.
13. Suryaningsih S. Karakter Morfometri dan Karakter Reproduksil kan Brek, *Puntius orphoides* (Valenciennes, 1842) dan Tawes, *P. javanicus* (Bleeker, 1863) di Sungai Klawing Purbalingga, Jawa Tengah. Disertasi. Universitas Gajah Mada. Yogyakarta; 2012.
14. Danish M, Singh IJ, Giri P, Singh CP. Molecular characterizaiion of two populations of catfish *Clarias batrachus* L. Using random amplified polymorphic DNA (RAPD) markers. African Journal of Biotechnology. 2012;11(7):14217–14226.
15. Fauzan AL. Karakteristik Genotipe Hibrida Hasil Persilangan 3 Strain Ikan Nila *Oreochromis niloticus* Dengan Metode Random Amplified Polymorphic DNA (RAPD). Skripsi FPIK IPB. Bogor; 2010.
16. Ikawikanti A, Padaga MC, Oktavianie DA. Isolasi Dan Karakterisasi Salmonella Spp. Pada Lingkungan Peternakan Ayam Broiler di Kota Malang. Program Studi Kedokteran Hewan. Universitas Brawijaya. Malang; 2012.
17. Ali BA, Ahmed MMM, El-Zaeem SY. Technical note: Application of RAPD markers in fish: Part II-Among and within families; Cichlidae (Freshwater), Mugilidae (Catadromous), Sparidae and Serranidae (Marine). International Journal of Biotechnology. 2004;6(4):393-401.
18. Suryaningsih S, Sagi M, Nitimulyo KH, Hadisusanto S. Beberapa aspek pemijahan ikan brek *Puntius orphoides* (Valenciennes, 1842) di Sungai Klawing Purbalingga, Jawa Tengah. Jurnal Iktiologi Indonesia. 2003;12(1):35-48.

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