Original Research Article

Genetic diversity analysis of PoeciliidaeFishusing RAPD-PCR method

ABSTRACT

There are many types of Poeciliidae fish in nature. Poeciliidae is a family of freshwater fish in the order Cyprinodontiformes and are well-known aquarium fish, such as guppies, mollies, platies and swordtails. Therefore, genetic analysis on Poeciliidae is important to collect information about biodiversity, genetic classification, and study developments that occurred during evolution. One approach to determine genetic diversity in many fish species can use Random Amplification Polymorphic DNA (RAPD). This study aims to analyze the genetic diversity of Poeciliidae fish using the RAPD-PCR method. This research was conducted at the Biotechnology Laboratory, Faculty of Fisheries and Marine Sciences, Padjadjaran University, Indonesia. The samples used were guppy fish (1), platy (2), molly (3), swordtail (4), and goldfish (5). RAPD-PCR was performed using OPA-6 primer. Procedures include sampling, isolation or extraction of DNA, DNA amplification using the RAPD technique, electrophoresis, and data analysis using NTSys. Genetic diversity of Poecillidae can be analyzed using RAPD-PCR. The phylogenetics of the Poeciliidae fish samples analyzed using OPA-6 showed that the guppy fish species (sample 1) are closely related to the platy fish (fish sample 2). Furthermore, the clade branched out with sample 3 (molly fish), and the three samples are related to sample 4 (swordtail fish).

Keywords: Genetic diversity, NTSys, Phylogenetics, Poeciliidae, RAPD-PCR

1. INTRODUCTION

Poeciliidae is a family of freshwater fish in the order Cyprinodontiformes, toothed fangs, and includes well-known live-bearing aquarium fish, such as guppies, mollies, platies, and swordtails. The family's original distribution is the Southeastern United States north of the Río de la Plata, Argentina, and Africa, including Madagascar (1). Currently, in Indonesia the existence of this fish is abundant. The spread of introduced/foreign fish into an area is one of the main causes of threats to fish diversity in nature (2–4).

Members of the Poeciliidae sub-family which are well-known in America as ornamental fish such as: guppies, swordtails, mosquito fishes, platys (5). Fish belonging to the guppy's group which are abundant in Indonesia include *Poecilia wingei, Poecilia velifera, Poecilia reticulata, Poecilia gracilis,*and *Poecilia cau*cana; swordtails fish including *Xiphophorus cuocinus, Xiphophorus montezumae*, and *Xiphophorus hellerii*, while the mollies fish group are *Poecilia mexicana, Poecilia sphenops* and *Poecilia latipuncata*(5). Diagnostic characters of fish from the Poeciliidae family include one dorsal fin with soft rays, A9, the third anal fin rays are not branched, the caudal fin is rounded or emarginated, V6, P9-16, sexual dimorphy in terms of body size and pattern color, and has cycloid scales (6).

One approach to determine genetic diversity and kinship in many species of fish is to use Random Amplification Polymorphic DNA (RAPD). Random Amplified Polymorphic DNA (RAPD) is a PCR technique that uses a random genome sequence. This technique has very high polymorphic ability in listing a genome. The RAPD code serves to study

genetic diversity in a species (7). The RAPD method is very effective and efficient in the current molecular field. The RAPD method is widely used in carrying out gene diversity in living things.

In addition, the RAPD method is also used to see the kinship of species with other species. Several studies have been carried out including the relationship between four strains of Guppy Fish (Poecilia reticulata)(7), variations of four species of ornamental fish family: Poecilidae (Order: Cyprinodontiform) (8) and other research. Therefore it is necessary to conduct research on the genetic diversity of Poliicidae fish as ornamental fish which are in great demand in everyday life. This research using RAPD-PCR method.

2. MATERIAL AND METHODS

2.1 MATERIAL

This research was conducted at the Biotechnology Laboratory, Faculty of Fisheries and Marine Sciences, UniversitasPadjadjaran.The tools used in this research consisted of : Aluminum foil, Analytical Balance, Autoclave, 100 ml Scott Bottle, Centrifuge Bottle, Petri Dish, Microcentrifuge, Agarose Mold- Tank, Electrophoresis, Coolbox, Funnel, Freezer (-20℃), Measuring Cup, Jerrycan, Cuvette, Microtube 1.5 ml, Microwave, Micropipette (0.5-10ul, 10-100ul), Microtips (volume 10 ul, 100 ul, dan 1000 ul),Plastic tray, Parafilm, Tweezers, Heat-resistant plastic, Plastic wrap, Power supply, Micro-tube rack, Refrigerator (4℃), Flat spoon, Spatula, Spectrophotometer, Plastic chopsticks, Thermal Cycler PCR, Timer, Vortex.

Meanwhile, the materials used consist of: distilled water, alcohol 95%, Bench Top DNA Ladder, 1 Kb (Marker), Ethano1 70%, Agarose Gel, Gel Red, GoTaq Green, Master Mix (Promega), fish (sample), Isopropanol, Cotton, Gauze, Tris, Borate, EDTA, (TBE), Loading Dye, Nuclease Free Water (NFW), Primer, Sterile Deionized Water (SDW), Wizard Genomic DNA Purification Kit.

2.2 METHODS

2.2.1 Sampling Method

The following is a method for taking fish samples by dissecting their fins and then observing them using surgical scissors. If the sample is not used immediately, the sample can be stored first by soaking it in a preservation solution (Alcohol: glycerol, 4:1). The sample stored at a maintained temperature (-20^{o}C).

2.2.2DNA Isolation

The following is a DNA isolation procedure (Promega): 20 mg of fish fins were put into a 1.5 mLmicrotube. Add 500 µl of Nucleic Lysis Solution until the cells were suspended and vortexes for 10 seconds. Cell lysis was incubated at 65°C for 30 minutes and then cooled to room temperature. Add 3 µl RNAse Solution to the lysis cell and stir until mixed. Incubate for 30 minutes at 7°C (cold temperature). Put 200 µl of Protein Precipitation Solution (PP) into the RNAse treated cell lysate then vortex at high speed for 20 minutes until the two solutions are mixed. Samples were incubated in ice for 5 minutes.

Samples were centrifuged at 13000-16000 xg for 4 minutes. Supernatant containing DNA was transferred into a new 1.5 mL microtube containing 300 µl isopropanol and then stirred slowly. Centrifuge at 13000-16000 xg for 1 minute. The supernatant was carefully removed and the tube was cleaned of absorbent. Add 600 µl 70% ethanol and carefully invert the tube to clean the DNA pellet. Centrifuge for 1 minute at a speed of 13000-16000

2.2.3 PCR

The amplification of the DNA of the tested fish in this study used OPA-06

(GGTCCCTGAC).RAPD-PCR reaction components and setting thermal cycle as listed in Table 1 and Table 2.

Table 1. Components of the RAPD-PCR Reaction

Reagent	Volume (µL)
MyTaq Master Mix	12,5
Water nuclease free	9,5
Primer RAPD (OPA-06)	1
Template (Sampel DNA)	2

Table 2. RAPD-PCR Program Settings

Cycle	Temperature (°C)	Time (second)	Number of Cycles
Pra Denaturasi	95	120	1
Denaturasi	94	60	45
Annealing	36	60	45
Extension	72	120	45

2.2.4 Electrophoresis

To determine the size of the molecules in a sample, known size standards are separated on the same gel and then compared with the sample(9). Several stages in electrophoresis are as follows:Prepare 0.4 grams of agarose powder gel;Put the powder into the Erlenmeyer or Schott bottle and add 40 ml of TAE; Heat the TAE solution which has been mixed with agarose powder until this solution boils using the microwave for two minutes; After that, let the agarose solution stand at a warm temperature, then add 0.06 µl red gel and homogenize the agarose, TAE and red gel; Prepare a complete agarose gel mold with a comb to print the wells on the agarose gel, then pour the agarose solution into the agarose mold. Let the agarose gel sit for 25-30 minutes or until frozen

After sufficiently frozen, insert the gel into the TAE running buffer solution by submarine;The results of the DNA amplification were filled in each well of the agarose gel with the composition of 4µl of yielded DNA and 2µl of loading dye, the other wells were filled with 2µl of 1kb DNA Ladder plus 2µl of loading dye as a marker; After all samples are inserted into the wells on the agarose gel, make sure the agarose gel is completely submerged in the TAE solution; The electrophoresis process lasted for 30 minutes at 80 volts; After the running process is complete, the agarose gel is taken and then placed on the UV Transilluminator machine; Electrophoresis results are documented using a digital camera for later analysis;The template DNA sample will be banded or striped when viewed with special glasses (anti-UV glasses) or when viewed in the dark it will glow.The electrophoretic method consists of several stages, namely: Enzyme extraction, Starch gel preparation, Sample placement, Electrophoretic process, Enzyme system visualization, Analysis method.

2.2.5 Data analysis of RAPD-PCR results

Data analysis of DNA amplification electrophoresis results were processed using CorelDraw X7 software so that the size of the DNA array could be analyzed specifically. Analysis was performed from the gel well down to standard DNA fragment sizes. Then a logarithmic value is made which is then used as the Y-axis. In Microsoft excel, a linear line equation is made y=ax+b from the migration distance of standard DNA and log pb on PCR markers. The size of the amplified DNA array (in bp) is searched by entering the value of the DNA migration distance (as the x value)(10).

The result of the y value is changed to an antilog, the result of the antilog shows the base size of the array. And finally, with the help of NTSYSpc 2.02, phenogram images will be obtained which will then be analyzed based on the images(11). Data were analyzed using a quantitative descriptive method. The descriptive method aims to describe what is seen or exists, in which there are efforts to describe, record results, analyze, and interpret the conditions that occur or exist. The quantitative approach is the analysis of research data using formulas and numbers or statistical methods. Quantitative descriptive is data obtained from a sample of the study population which is analyzed according to the statistical method used.

3. RESULTS AND DISCUSSION

Genomic DNA isolation applies procedures and methods according to the Genomic DNA Purification Kit Wizard, also known as the Promega Kit. The body part of the fish that was sampled was the tail of the fish. The tail is the body part of the fish that is most often actively moved in water and requires energy to regenerate its muscle cells. This part of the tail is used as a sample in order to get parts that can represent cells in the body of the fish used as test samples which are in good condition. In addition to this, the tail part that is used as a sample can also be taken directly without having to kill the fish.

The genomic DNA obtained from the isolation was then tested quantitatively by using a spectrophotometer to measure the purity of the sample DNA. The concentration of good DNA quality ranges from 1-10 µg/µl. The purity level of pure DNA isolates has a value in the range of 1.8-2.0 and was tested qualitatively by applying the identification, separation and purification of DNA fragments using agarose gel electrophoresis. When the level of purity is low, the primer attachment will be disrupted and the activity of the DNA polymerase enzyme will be inhibited(12).

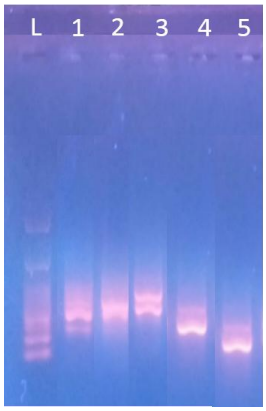

Figure 1.Results of PCR-RAPD

Description = L : ladder, 1 : guppies, 2 : platty fish, 3 : molly fish, 4 : swordtail fish, 5 : goldfish

The results of the genomic DNA (Figure 1) amplification process obtained showed the presence of band fragments from the DNA produced by the OPA-06 primer. These fragments are produced in a variety of ways due to differences in the nucleotide sequence of the primer attachment sites(13). The amplified band really depends on the quantity, quality, and suitability of the venue and the primer used. These primary attachment sites are randomly distributed throughout the genome, which then polymorphism in this area will result in different amplifications(14).

Band fragments of RAPD are formed due to nucleotide substitution which then creates or removes the primary attachment site or insertion. And deletions in the area between primers can affect the resulting fragment size changes. The band fragments formed are divided into two groups, namely polymorphic bands and monomorphic bands. The polymorphic bands are a description of DNA bands at a certain size, but when seen in other test fish no DNA bands with that size are found. And monomorphic bands are bands that exist in several test fish and therefore, there is no variation in this type. The bands amplified by the OPA-06 primer range from those presented in the Table 3.

Table3. Sample Fragment Distance

Fragment Distance (bp)	1	2	3	4	5
2.475			*		
2.965	-	-	-		
3.241	-			-	
3.243					*
3.387					*
3.391					*

Description =1 : guppies, 2 : platty fish, 3 : molly fish, 4 : swordtail fish, 5 : goldfish

Based on the Table 3, goldfish have the most amplified bands when compared to other samples when using the OPA-06 primer. There are 2 DNA bands in guppies, 1 DNA band in platty fish, 2 bands in molly fish, 1 band in swordtail fish, and 3 DNA bands in goldfish. There are polymorphic bands in goldfish and molly fish. The four bands were not found in other sample test fish. The size of the molly fish polymorphic DNA band is within 2,475 bp, the polymorphic band of 3,243 bp is found in goldfish, the polymorphic band of 3,387 bp is found in goldfish and the last polymorphic band at 3,391 bp is also in goldfish. The existence of these polymorphic differences is possible as a result of the expression of visible traits or quantitative phenotypes of the test fish. Or this is also triggered by the invisible nature of the sample fish, usually in the form of growth speed, immunity, total fecundity, or other factors or characteristics of the fish(15).

A phylogenetic tree, also known as phylogeny, is a diagram that depicts the evolutionary lineage of different species, organisms, or genes from a common ancestor. Phylogeny is useful for organizing knowledge about biodiversity, for constructing classifications, and for providing insight into events that occurred during evolution(16). Moreover, because these trees show descent from a common ancestor, and because much of the strongest evidence for evolution comes in the form of a common ancestor, one must understand phylogeny to fully understand the evidence supporting the theory of evolution(17).

The phenogram obtained from the bands that appear in the OPA-06 primer is then converted into a binary matrix form which is then processed again by adapting the NTSYS program to produce a family tree from the tested fish samples. The results of the phenogram amplification of NTSYS are shown in the following Figure2.

Comment [GdG1]: I suggest that the authors carry out a statistical analysis of the data with measures of central tendency and dispersion

Comment [GdG2]: Make a boxplot with 1 : guppies, 2 : platty fish, 3 : molly fish, 4 : swordtail fish, 5 : goldfish, so we can see the variation between the fish.

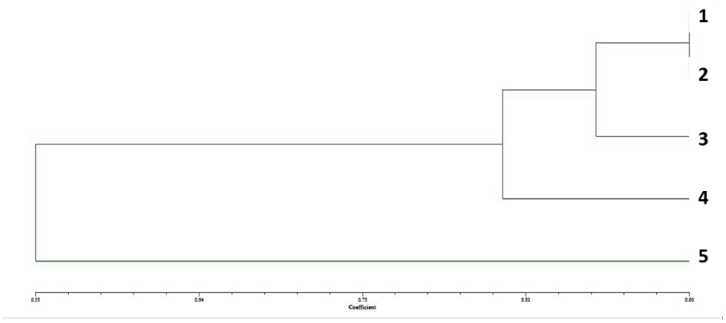

Figure 2. NTSYS Phylogenetic Tree
Description =1 : guppies, 2 : platty fish, 3 : molly fish, 4 : swordtail fish, 5 : goldfish

Comment [GdG3]: Dear authors, you could improve the quality of the figure because it is very small

Is figure 2 a dendrogram?

Based on the phylogenetic results that were processed using NTSYS (Figure 2), it can be seen that the Poeciliidae Fish sample is divided into two main branches. Where the first branch consists of 2 main branches (upper part) then has 2 more sub-branches. It can be seen the fish species that are most closely related are sample 1 (guppies) and sample 2 (platty fish). Furthermore, Clade branched out with sample 3(molly fish). The three samples are related to 4 (swordtail fish). All 4 samples 1 (guppies), 2 (platty fish), 3 (molly fish) 4 (swordtail fish)were outgrouped with sample 5 (goldfish), which are fish of the family Cyprinidae.

Comment [GdG4]: What is the cut line (phenon line) in the dendrogram showing ? What method is used in the dendrogram?

The phylogenetic tree that was formed obtained that the sample 1 (guppies), 2 (platty fish), 3 (molly fish) 4 (swordtail fish) one Poeciliidae ancestor. In contrast to sample 5 (goldfish) which is an outgroupfar apart in the phylogeny tree. This is due to goldfish is part of Cyprinidae.Based on the results that have been described, the value of genetic distance is the level of gene difference between populations or species(18). The higher the value of genetic distance, the species has a more distant kinship. Conversely, if the lower the value of genetic distance, the species has a closer kinship(19). High kinship is due to the same color, body shape, and head size, while distant kinship is due to the offspring of the same fish to brooders which are taxonomically different(20).

These results can be affected by the number of chromosomes in an individual.The number of chromosomes in a set of each species under normal circumstances is fixed. Although the number of chromosomes of one species may be the same as that of another species, they differ in the shape, size and composition of their genes (21). The further the kinship of an organism, the greater the possibility of differences in the number, shape and arrangement of its chromosomes (21).

4. CONCLUSION

Poecillidae genetic diversity can be analyzed using RAPD-PCR. The phylogenetics of the Poeciliidae fish samples analyzed using OPA-6 showed that the guppy fish species (sample 1) are closely related to the platy fish (fish sample 2). Furthermore, the clade branched out with sample 3 (molly fish), and the three samples are related to sample 4 (swordtail fish).All 4 samples 1 (guppies), 2 (platty fish), 3 (molly fish) 4 (swordtail fish)were outgrouped with sample 5 (goldfish), which are fish of the family Cyprinidae.

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