

Genetic Diversity and Geographical Gene Flow Patterns of Spawning Broadcast Coral *Lobophyllia corymbosa* in The Sulawesi Waters as A Coral Triangle Area

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Abstract. The existence of hard coral is one of the keys to maintain the sustainability of ecosystem in the waters. Currently, the hard coral keeps declining over time due to various disturbances. In addition, hard coral where fish and other organisms live in will directly affect the ecosystem sustainability if the damage still happens. Molecular approach, such as knowing the genetic variation information of coral population can be an informative study to estimate the condition of waters, so that, conservation efforts can be easily done. In this study, we use *Lobophyllia corymbosa* as a spawning broadcast coral to be the sample which is collected from Sinjai and Luwuk Banggai. The study areas are selected based on geographical patterns which are in the South and East of Sulawesi waters. Since they have a considerable distance, it is likely for them to produce high genetic variations. Genome DNA uses mitochondrial genome that is extracted from coral tissue. The result shows that the genetic diversity is high. From the two major groups provided, there have been 8 haplotypes for all locations. In addition, the *L.corymbosa* distribution between Sinjai and Luwuk banggai has a high genetic connectivity with 0.6 fixation index .

Keywords : Genetic Populations, Gene Flow, Hard coral, Conservation Management, *Lobophylliacorymbosa*.

1. Introduction

Genetic variation is a condition when there is genetic diversity in a species, either between the separated population geographically or individual in a population. The genetic variation found in an individual is closely related to the kinship that occurs in the same individual but geographically separated. In this case, biological connectivity that occurs in an organism can be used as a basis in maintaining population and recovering from damages [1]. Biological conservation by looking at the genetic relationship of an organism is based on several main theories of population spawning, genetic origin of organisms, biogeography, and distribution patterns [25]. Besides that, [16] given the same states that the pattern of relationship of an organism is influenced by several factors, such as reproduction, larval distribution, geographical patterns, and physical oceanography.



Coral is one of the aquatic organisms with a very important role. Nowadays, the existence of coral is increasingly threatened, the damage has become worse which are caused by anthropogenic and natural factors. Could not be denied that the condition occurs in the organism are sixty percent caused by the activities carried out by humans in the water either directly or indirectly [12]. In addition, for coral conditions in the World's Coral Triangle Area, experiencing a higher threat compared to globally, the threat of anthropogenic activity as much as 85% is experienced in the region as the world's highest provider of marine biodiversity [5]. One of the hard corals needed to be preserved today is *Lobophyllia corymbosa*, since it is a kind of ornamental corals that have been widely exported and traded. Based on the data published by the International Union for Conservation of Nature (IUCN) in 2014 [15] which states that *L.corymbosa* species into the vulnerable organism. Seeing the pattern of coral distribution in a waters will provide information about the ability of the organism in adapting to an environment, at least genetic variation is directly related to the ability of an organism to live in a waters [9, 11] Reproduction of *L.corymbosa* that occurs sexually with external fertilization [6] may support the possibility of genetic connectivity in any population of *L.corymbosa* coral or other population with the same reproductive system. It is based on a longer-term developmental phase in the water columns compared to internal fertilized reproducing, so that, the duration during the settlement process of coral larvae will be longer and the spread triggered by current movement will become very easy to happen. The distance of the island and the area contained in Indonesia in general can be a transit area of a larva in its migration until it is finally settle to the suitable substrate. The current oceanographic factor plays a major role in the migration of larvae. Coral life cycles with limited mobility in the larval phase make the movement of these organisms strongly influenced by oceanographic conditions occurred in waters. Wherein its planktonic phase, the larvae migrate follows the movement of the current waters from the hatching site to the area to be alive and growing, and this displacement can take place within a few meters to hundreds of kilometers depending on the current velocity at a site.

The former research [2] has found *Acropora* sp., a species collected from Caribbean waters, geographically distributed as far as 500km and it is seen through a genetic approach. Coral population in Sulawesi waters has a high diversity of organisms, especially in the Sinjai and Luwuk Banggai areas as a Coral Triangle Area. Geographically, the distance from these two areas is \pm 700km. This research will look at *L.corymbosa* distribution and genetic variation based on geographical location, so it can be predicted for other hard coral species that have the same reproductive system.

2. Research Methods

This research conducted in Sulawesi waters is done by purposive random sampling. Sample of hard coral *Lobophyllia corymbosa* species were taken using hammer and chisel along 3-7 cm as many as 25-30 coral fragments. We collected the coral sample in a depth of 3-7 meters in Sinjai and Luwuk Banggai as a geographically separated area (Fig.1). The coral fragments that have been taken will be preserved into 96% alcohol and fed into plastic containers for subsequent transport to the laboratory. Alcohol replacement is performed when the sample has been in the laboratory using 100% absolute ethanol which is then stored at room temperature [14].

In this study, there are several stages of the procedure to be performed, including as follows ;

2.1. Molecular analyses (mtDNA)

In this study the sequenced genome is mitochondrial DNA or mtDNA to the coral, at this stage performed several stages in the molecular analysis procedure that begins with DNA genome extraction, PCR (Polymerase Chain Reaction), electrophoresis and sequencing.

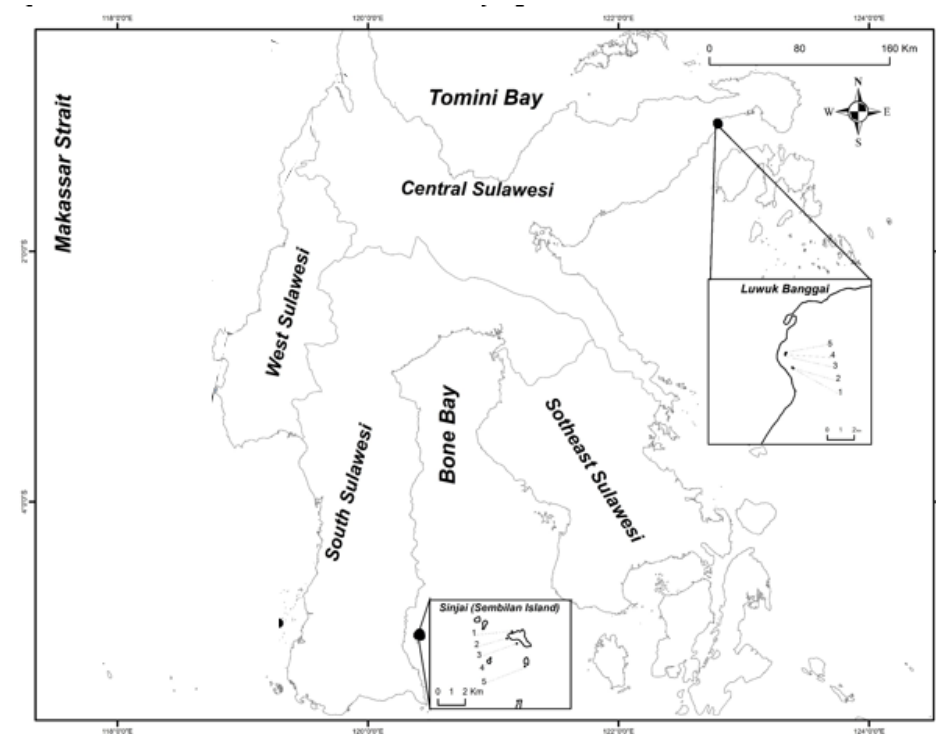


Figure 1. Map of research sites in Sulawesi waters (There are from Sinjai and Luwuk Banggai).

2.1.1. Extraction

DNA genome extraction was performed using an extraction kit (Qiagen Kit), DNA extracted the following the previous research [14]. The extraction process carried out in principle is the same as network retrieval in the sample. At this stage, coral tissue retrieval by crushing fragments of coral samples that have been collected and weighing 0.10 grams, Then, put in a tube of 1.5 ml. After that, the tissue was given an ATL Buffer solution of 180 μ l and added with 20 μ l Proteinase K. The second solution was homogenized using a vortex for 15 seconds, then incubated in a heat block at 56 °C for 24 hours (over night) to obtain a good lysis result.

The result of lysis has been obtained, then re-vortex before the next reagent. After that, the ALL Buffer is 200 μ l, then added with 200 μ l ethanol 96%. All reagents that have been mixed always end with stirring using vortex. Furthermore, the supernatant fluid on the tube was removed in a 2 ml Dneasy mini spin column which was then centrifuged for 1 minute at a speed of about 8000 rpm.

At this stage, there is a filtered network on the mini-spin filter paper. The escaped liquid is subsequently discarded along with the tube. Replacement of tube is performed for subsequent reagents. Mini spin column that has been placed on the new tube then given a solution of Buffer AW 1 as much as 500 μ l and re-centrifuged for 1 minute at a speed of 8000 rpm. Next, add the Buffer AW 2 solution and centrifuged for 3 minutes at a speed of 14 rpm. For good results, repeat the centrifuge process for 1 minute at 8000 rpm. Then, remove the remaining liquid filter along with it's tube. After that, the spin column was transferred into a 1.5 ml tube which was then added with 100 μ l ddH₂O for DNA elution. The next liquid is incubated at room temperature for 1 minute and then centrifuged at 8000 rpm in 1 minute. This separation phase of DNA (elution) is repeated once so the volume of DNA template obtained is 200 μ l. The extraction results are then preserved in a freezer at -4°C.

2.1.2 Polymerase Chain Reaction (PCR)

PCR is a technique in the stages of molecular work that serves to replicate DNA enzymatically without the aid of the organism. In this process, the reagents used are MyTaq RedMix (Bioline). Where the reagents contained therein are mixed in one container such as Enzyme, MgCl₂,

PCR Buffer and others. At this stage, primer selection is carried out, referring to the ascension numbers contained on the NCBI Web (National Center for Biotechnology Information), specific mitochondrial COI primers can be designed and in BLAST to be suitable for the target sample. The DNA base order obtained for the primers in this sample is mtCOIF CAGGCGCTATGTTAGGAGATG as a forward and mtCOIR CCCGCTAATACAGGCAAAGATA as a reverse. Primer selection is intended to find a primer that can be amplified, because the success at this stage is based on the primary suitability used.

The first thing to do in the PCR process is to prepare 1.5 ml tube as a master mix to mix ddH₂O, primer forward and reverse and reagent in one container then mix. Next, the reagent on the master mixed tube was inserted into a 24 µl PCR strip and added 2 µl of a DNA template. Furthermore, the mixing results are spin down in order that there is no bubble and then inserted into the PCR machine with the machine settings referring to the basic protocol to amplify the mitochondrial genome based on research [10]. Then, were performed 38 cycles, each cycle, pre-denatured with 95°C for 120 s, denaturation at 94 °C for 45 seconds, annealing or pasting at 53°C for 30 seconds and extending at 72°C for 90 seconds and Final stage at 72 °C for 5 minutes.

2.1.3. Electrophoresis

Success or failure of PCR process can be known by visualizing PCR products through electrophoresis process. The first stage of electrophoresis is making of 1% agarose gel by 0.75 gram using SB Buffer solvent as much as 75 ml. The solution is entered into a beaker glass which is then cooked using a microwave for 3 minutes or until the agarose powder has dissolved completely. Thereafter, Ethidium Bromide (4 µl) dye was added and then poured into a combed mold and allowed to stand for 15-20 minutes. Subsequently, 4 µl of the product The PCR product was taken and mixed with the Loading dye (1 µl), then inserted in the well in the agarose mold submerged in the SB Buffer solution. Furthermore, a marker added to the well that does not contain PCR products, as a marker in view of the location of the DNA band at the time on visualization steps. Electrophoresis was performed at a voltage of 100 V, 400 Amp for 30 min. The band of electrophoresis can be seen by using ultraviolet light on UV transilluminator. Positive PCR results contain the desired DNA, then sent to the Sekuensing Center, Berkeley. DNA sequencing is a technique for determining the sequence of nucleotide bases in DNA molecules, it aims to determine the genetic identity. The principle of this sequence is to determine the order of base ACGT used as a template for later amplified using enzymes [27].

2.2. Data analyses

To achieve the research purpose, there are some data analysis process to be done, referring from the data analysis that has been used in the previous researches.

2.2.1. Gene flow (Genetic Connectivity) and Haplotype Network

Sequencing results have been obtained, then the results are sorted and edited by using MEGA 5.05 software (Molecular Evolutionary Genetic Analysis) [24]. The edited data is then analyzed by the AMOVA (Analysis Molecular of Variance) program found in Arlequin [7]. Analyze the data to obtain the Fixation Index value and the genetic population structure of the coral sample.

In addition, to see the haplotype network in both populations, we used Network 5.0.0.1 software, so that, the haplotype composition will view in each population is presented in the form of connected diagrams.

2.2.2. Genetic Diversity

The diversity of haplotypes and the diversity of nucleotides [18] in the *L.corymbosa* sample, was measured using DNAsp software 5.0 [22], the values obtained in the analysis are then incorporated into the categories stated by Nei [21] in Table 1.

Table 1. Category index of Genetic diversity [21].

Anlaysis	Low	Medium	High
Genetic Diversity	0.1-0.4	0.5-0.7	0.8-1.0

3. Result and Discussion

3.1. Gene flow and Genetic Patterns

The sequencing product had more than 500 bp and after removing and editing the base nucleotides considered to be ambiguous long bases to 450 bp, the mitochondrial genome derived from 60 total samples taken from Sinjai and Luwuk Banggai Waters (each 30 samples per population). Sixty samples, only 30 were successfully amplified and produced an informative sequencing product. Specimens from Luwuk Banggai waters have 10 detected polymorphic sites, which produce 4 haplotypes. These polymorphisms included 9 transitions and 1 transversion. While in Sinjai waters, detected polymorphic sites are 9 with 4 haplotypes. Polymorphisms consist of 8 transitions and 1 transversion. The results of all samples obtained have a similarity about 98% -100% with *L.corymbosa* species on Genbank with an accession number AB117241 [10].

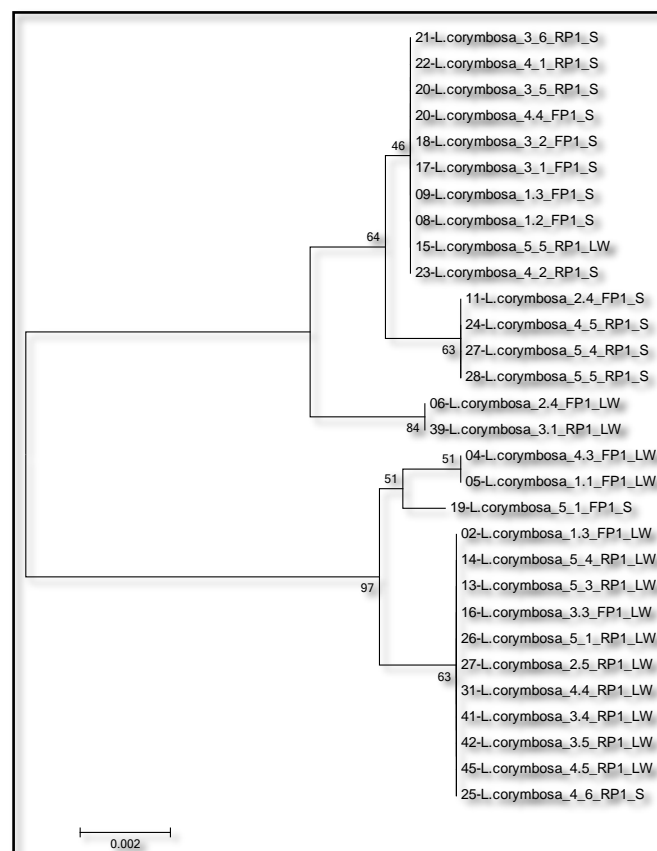


Figure 2. Neighbour-joining tree of mtDNA. Bootstrap values for Sinjai (S) and Luwuk Banggai (LW) are shown above branches.

When it is viewed from the tree phylogeny shown in Figure 1, formed 2 large clusters are divided into several groups. In the first cluster, the cluster branch shows the existence of gene contributions from the Luwuk Banggai population incorporated in a large section of the Sinjai population group, and vice versa. Results demonstrated by phylogeny trees, supporting the value of

genetic distance analysis within and between populations of *L.corymbosa* (Table 3) at Luwuk Banggai and Sinjai. The genetic distance is the value that shows the genetic relationship found in a population. In this study, the genetic distance values of both populations fall into the low category based on the value determined by [20] which states that, the lower the genetic distance value in a population indicates that there is a very close genetic relationship in the population. The genetic distance in the population from 2 sampling sites is 0.003 and for the inter population is 0.007. The low of value, indicating that the current flow in both of locations still affect each other.

Sinjai waters entering the Bone Bay area are the waters that are still directly influenced by the current factor comes from Makassar Strait and Flores Sea. The absence of spawning recording data on spawning period of *L.corymbosa* specifically makes predictions of larval distribution of the reef a little difficult. However, if the referring is based on the constant reproduction of reefs that occur in tropical waters, especially on hard corals that reproduce sexually externally (broadcast spawning coral) so that spawning cycle occurs on an annual basis (Single Gametogenic Cycle) and generally reproduce in August – December [13]. The period of reproduction time is directly related to West Monsoon Wind period occurring at the time of spawning, especially in the waters of Sulawesi. In October - April, the wind blows from the Continent of Asia to the Continent of Australia, thus, the movement of the wind that affects the surface currents that occur in the Sulawesi waters blow from North to South Sulawesi waters and receive the flow of Flores Sea flows so it turns to the left (East) Sulawesi Waters. This current flow makes the possibility for the Sinjai and Luwuk Banggai Waters to share genes, because according to expert [4] that current circulation patterns can provide genetic information from an organism that has a dispersion that depends on the movement of the current.

Table 2. Hierarchical analysis of molecular variance (AMOVA) that packaged in Arlequin ver 3.5.2 of mtDNA.

Source of Variation	d.f	Sum of Squares	Variance components	Percentage of Variation
Among Population	1	29.267	1.862 Va	58.09
Within Population	28	37.600	1.343 Vb	41.91
Total	29	66.867	3.204	
Fixation Index	Fst	0.581		

The value of genetic distance obtained and included in the Nei's categories [20], the category has an index that is directly proportional to the Fixation Index category (Fst) that obtained in the entire population. The value of Fst or the value of population structure is useful to provide a genetic interpretation that shows the genetic flow in a population. Coral population of *L.corymbosa* in Sinjai and Luwuk Banggai showed Fst value of 0.581 or 0.6 (Table 2). [8] further explains that genetic flows that are in the range of 0.6-1.00 fall into the high category. Thus, it can be said that the greater the value of the genetic flow, the greater of genetic flow entering a population. The genetic flow rates obtained at both sites provide information that there is a close familial relationship that may represent genetic connectivity at each location.

Based on the patterns of genetic connectivity obtained from both populations, it can be clearly assumed that there is a high biological linkage to the population. However, if viewed directly at the *L.corymbosa* coral sampling site, these coral waters are classified at an alarming rate, where anthropogenic activities that tend to be damaging are still frequently performed which can threaten sustainability or even break the genetic network of the coral species as well other. According to [19] spatial and temporal connectivity can be used as an implication of coral reef ecosystem management, particularly in designing marine conservation areas. However, if these threats persist, there is no possibility of causing genetic flow termination in both populations.

Table 3. Estimates of Average Evolutionary Divergence over Sequence Pairs Within and Among Groups. (The number of base substitutions per site from averaging over all sequence pairs within each group are shown. Analyses were conducted using the Maximum Composite Likelihood model [23]. The analysis involved 30 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 450 positions in the final dataset.

Genetic Distance <i>D</i>	Location	Luwuk Banggai	Sinjai
Within population	Luwuk Banggai	0.003	-
	Sinjai	-	0.003
Among Population	Luwuk Banggai	-	-
	Sinjai	0.007	-

3.2. Genetic Diversity

The results of analysis performed with DNAsp software, obtained 8 haplotype (*h*) for the entire population (Table 4). The population of Sinjai waters has 4 haplotypes, as well as Luwuk Banggai's performances of 30 total samples amplified. In addition, for genetic diversity in each population showed a level of haplotypes diversity (*Hd*) in the medium category ranged from 0.552-0.600 based on Nei classification in 1987 [21]. As for the nucleotide diversity (π) ranged from 0.005-0.007.

Table 4. Genetic diversity pututative population of *Lobophyllia corymbosa*.

Site	Specimen	Number Of Haplotype <i>h</i>	Haplotype diversity <i>Hd</i>	Nucelotida diversity π	Mean pairwise difference	Number of sites with substitutions <i>S</i>
Luwuk Banggai	30	4	0,552	0,007	3,030±1,670	10
Sinjai	30	4	0,600	0,005	2,343±1,354	9

The high genetic diversity/haplotype values in a population reflect the size of the population in an area, while the decrease in population size will reduce genetic diversity or haplotype diversity. The genetic diversity found in Luwuk Banggai and Sinjai is still categorized in the average population size. However, the low genetic diversity category ranges from 0.1-0.4 [21] where, both populations tend to be threatened for impairment, if destructive anthropogenic activity is still common in these waters. One study conducted [26] on the soft coral species *Scleronephthya gracillium* shows the environmental stress that occurs in the coral resulting in a change in gene expression in response to environmental changes and damage. If the change is not lethal and is passed on to the next generation it will increase the variation within the population, however, if the change is lethal it will decrease the population size. Genetic diversity provides the ability for an organism to adapt to environmental, climate change and disease [17]. Therefore, the genetic diversity in a population is a great capital for growth, development and regeneration.

Connectivity is closely related to genetic diversity. A well-preserved genetic connectivity will affect the high genetic diversity formed in a population. According to statement from the expert [3] the genetic diversity of a population will increase if there is genetic input from other populations or commonly referred to as genetic migration and that is the case in the waters of Luwuk Banggai and Sinjai (Figure 3). This is because a large migration will make in cross-breeding and mixing of genes between different populations, so that different gene variations can be obtained and may provide information on migrating pathways of coral larvae during the spawning season. Therefore, it is hoped that there will be special attention to efforts to improve the water quality in Indonesia, one of which is Sulawesi as the center of the world's coral triangle. The preservation of waters, it will also maintain genetic susceptibility to an organism and will also preserve the biodiversity of Indonesia, because genetic is the most basic and also smallest level of biodiversity for a conservation.

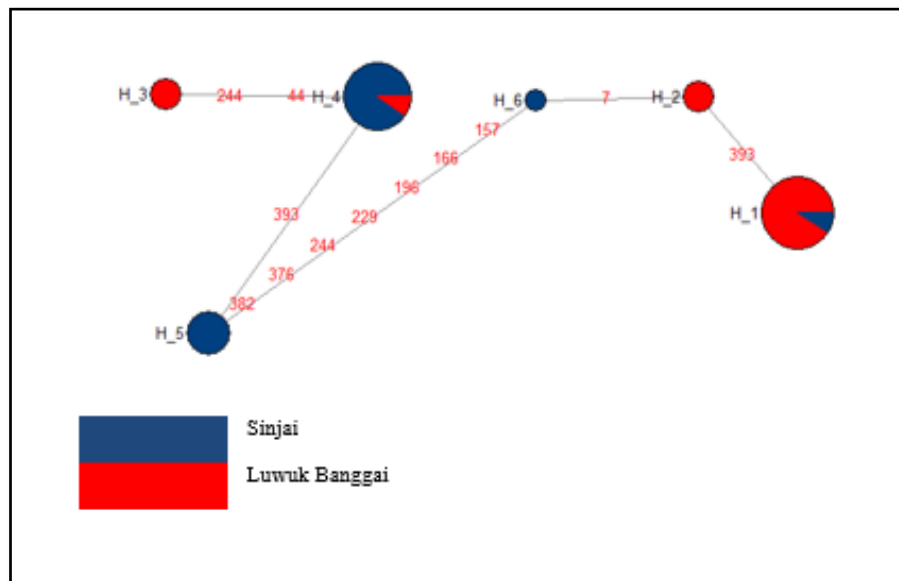


Figure 3. Haplotype network of native (n1 – n6), mtDNA haplotypes found in Sinjai and Luwuk Banggai.

4. Conclusion

The genetic diversity in Luwuk Banggai and Sinjai waters is in the high categories, but, tends to decrease in population size and based on genetic distance values and genetic flows of these two populations have high relation or connectivity and genetic mixing between them. So, the spacing between them is not an obstacle to the occurrence of genetic migration supported by the circulation of currents to connecting them.

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