Genetic differences and population structure of spotted barb (cyprinidae) collected from three rivers in Java Island

S S Astuti1, A M Hariati2, W E Kusuma2,3, D G R Wiadnya*

1Doctoral Programme of Fisheries Science and Marine, Universitas Brawijaya, Malang, Indonesia

2Department of Aquaculture, Faculty of Fisheries and Marine Science, Universitas Brawijaya, Malang, Indonesia

3Ichthyofauna, Faculty of Fisheries and Marine Science, Universitas Brawijaya, Malang, Indonesia.

*Corresponding author: dgr_wiadnya@ub.ac.id

Abstract. Barbodes binotatus is an endemic species and most dominant freshwater fish in Indonesia. This preliminary study aimed to calculate genetic differences of the species from three different rivers in Java island. A total of 90 specimens were collected from each river and a total of 26 sequences were analyzed using mitochondrial DNA (mtDNA) region Cytochrome Oxidase Subunit I (COI). BLAST analysis showed > 97 % similarity of B. binotatus with another research. The result of sequence analysis showed that B. binotatus from Central Java were not significantly different from B. binotatus from East Java, and B. binotatus from West Java were significantly different from two others. The genetic differences of B. binotatus from three locations were observed in five basepair (335 bp, 399 bp, 449 bp, 497 bp, and 545 bp). The result of population structure analysis showed an FST value of 1.00. It suggested that there was genetic differentiation between populations of Pasuruan, Yogyakarta and Cirebon. Inter-location analysis requires to be done to confirm the genetic differences from another regions.

1. Introduction
Barbodes binotatus is a species of freshwater fish that belongs to the genus Barbodes composed of 44 species. A total of 10 species out of 44 species of Barbodes are found in Indonesia, one of them known as spotted barb [1]. B. binotatus is also known as spotted barb which has two pairs of barbels on the sides of its mouth [2]. B. binotatus has characteristics that can be distinguished morphologically from other species, including : (1) body shape: lateral fusiform or elongate and vertical compress, straight dorsal head and terminal mouth position with 4 (four) grooves, number of scales between nape and dorsal 8- 9; (2) lateral line: complete, the number of scales on the lateral line 22-27, the number of scales above the lateral line 3.0-4.5 lines, the number of scales below the lateral line 2.5-3.0 lines; (3) dorsal fin: single, 4-4 hard spines, 8-8 soft spines, last dorsal fin soft thorns hardened and jagged back, no adipose fin; (4) caudal fin: forked and normal; (5) anal fin: single, hard spines 3-3, soft spines 5-5; (5) pectoral fin: hard spines 1, soft spines 14-15, (6) pelvic fin: abdominal position, (just below D1), hard spines 1, soft spines 8-8; (7) special characters: one spot (like a stem) on the base (anterior) dorsal fin,
one spot (round) on the caudal peduncle, on the juvenile (2-4) midlateral spots that are round or elongate, one spot (bar shape) at the end of the gill cover; (8) body color: varies from gray silver to gray green, dorsal dark green, whitish throat and pale abdomen [3].

*B. binotatus* is synonymous with *Systomus binotatus*, *Puntius binotatus* and *Capoeta binotata*. The morphological analysis of *B. binotatus* has various constraints because of the similarity of this species to several others. Genetic approach to this species is needed to evaluate the taxa of this species [4]. The identification of organisms with a molecular approach provides another alternative in the process of identifying organisms to decrease ambiguity and differences to the species level. Molecular approaches are widely used to identify populations within a species, for example: genetic diversity, phylogenetic and distribution of organisms [5]. The use of molecular identification approaches with DNA analysis makes it easy to analyze the relationship between interspecies and intraspesies of each population [6]. The development of research on genetic molecular with mitochondrial genome as one of the parts is capable to provide genetic information in analyzing the differences of any organism [7]. Mitochondrial DNA is widely applied, especially in phylogenetic studies [8]. Mitochondrial DNA in phylogenetic studies can be used to determine the differences between related species [9]. The most commonly used mitochondrial gene sequence is COI (Cytochrome Oxydase Subunit I) because of its suitability for estimating different taxa [10]. An understanding of the genetic diversity and population structure of organisms is useful to determine the management of fisheries resources [11]. Research on genetic diversity in a population has a function to determine the rate of genetic transfer between populations as a reference in determining the life status or conservation status of the organism population [12]. Populations with a high level of diversity tend to survive in their environmental life. Populations with low levels of diversity will be difficult to survive. In general, the degree of genetic diversity is also related to the size of the population [13].

2. Methods

2.1. Sampling and Fish Collection

A total of 90 specimens were taken from three different locations namely Tawang River (East Java), Opak River (Central Java) and Cisanggarung River (West Java), Indonesia. Specimens were captured with various types of fishing gear including nets and trap equipment. The specimens stored in 96% alcohol and labeled from each location. Fish specimens were submitted into the Brawijaya Ichtyologycum Depository (Figure 1). Fish tissue is taken from the right pectoral fin. Sample preservation was carried out by inserting it into the TNES buffer. Preserved tissue was stored at room temperature (27 °C).

![Figure 1. Barbodes binotatus (Specimen Collection)](image)
The location of sampling activity is based on geographic boundaries of fish distribution and river flow to ensure that the area is different from others (Table 1). Location marking of this research based on google earth and GPS. Three rivers were used as sampling areas in this preliminary study (Sungai Tawang, Sungai Opak, Sungai Cisanggarung).

### Table 1. Sampling Location

| No. | Location          | Specimen | Coordinate       |
|-----|-------------------|----------|-----------------|
| 1   | S. Tawang         | 30       | 7°38'53" S      |
|     |                   |          | 112°41'21" T    |
| 2   | S. Opak           | 30       | 7°53'33" S      |
|     |                   |          | 110°23'45" T    |
| 3   | S. Cisanggarung   | 30       | 6°50'29" S      |
|     |                   |          | 108°35'51" T    |

Morphological characters were carried out by identifying 11 characteristics of *B. binotatus*. Morphological analysis was carried out to determine the correctness of the species taken as research material. Morphological analysis is also useful to determine the population in each region (Table 2).

### Table 2. Morphological Character

| No.  | Parameter     | East Java                      | Central Java                     | West Java                     |
|------|---------------|--------------------------------|----------------------------------|-------------------------------|
| 1    | Body shape    | Fusiform/ Normal               | Fusiform/Normal                  | Fusiform/Normal               |
| 3    | Mouth         | Terminal                       | Terminal                         | Terminal                      |
| 4    | Fin Shape     | Forked                         | Forked                           | Forked                        |
| 5    | Pectoral Fins | Present                        | Present                          | Present                       |
| 6    | Dorsal Fins   | Present                        | Present                          | Present                       |
| 7    | Cudal Fins    | Present                        | Present                          | Present                       |
| 8    | Pelvic Fins   | Present                        | Present                          | Present                       |
| 9    | *Barb*        | Present                        | Present                          | Present                       |
| 10   | Anal Fins     | Present                        | Present                          | Present                       |
| 11   | Types of Scales | Sikloid                      | Sikloid                          | Sikloid                       |

2.2. DNA Extraction and Sequencing Method

Molecular identification carried out with several steps including extraction, amplification, electrophoresis, and sequencing. DNA was extracted from the tissue using phenol-chloroform method. A fragment of mitochondrial DNA (COI) was amplified using the primer of forward and reverse with Fish F1 [5'-TCAACCAACCACAAAGACATTGGCAC-3'] and Fish R1 [5'-TAGACTTCTGGTGCCAAAGAATCA-3'] [14]. A total of 50 µl contained with 3.6 µl of ddH2O, 2 µl of each primer (10 µM), 2 µl DNA template, and 5 µl Gotaq DNA polymerase. PCR products were checked using 1.5% agarose gel electrophoresis. The BLAST (Basic Local Alignment Search Tools) method is needed to identify similarities between species listed on GenBank.

2.3. Data Analysis

A fragment of 665 bp of COI gene was obtained from the specimens. Haplotype diversity and nucleotide diversity were calculated using Arlequin version 3.5 [15]. The phylogenetic tree was identified using MEGA software version 4.0 [16].

3. Result and Discussions

A total of 90 specimens were counted with six morphological characters and identified as *Barbodes binotatus* from the sampling area (Table 3).
The development of genetic markers makes it easy to detect genetic structures in each individual, population and species [17] [18] [19]. The results show that there are genetic differences in each sample taken in East Java, Central Java and West Java. The resulting difference is in the nucleotide base strands with base lengths of 335 bp, 399 bp, 449 bp, 497 bp and 545 bp.

| Characteristic                  | S. Tawang (N=30) | S. Opak (N=30) | S. Cisanggarung (N=30) |
|--------------------------------|------------------|----------------|------------------------|
| SL (Standart Length)           | 5.581 ± 1.000    | 3.478 ± 0.840  | 4.808 ± 1.095          |
| TL (Total Length)              | 6.978 ± 1.250    | 6.978 ± 0.800  | 6.098 ± 1.204          |
| BD (Body Depth)                | 1.636 ± 1.636    | 1.032 ± 0.420  | 1.371 ± 0.556          |
| PDL (Pre Dorsal Length)        | 2.88 ± 0.505     | 1.824 ± 0.530  | 2.498 ± 0.762          |
| PAL (Pre Anal Length)          | 3.93 ± 0.704     | 2.463 ± 0.640  | 3.394 ± 0.916          |
| PPL (Pre Pectoral Length)      | 2.652 ± 0.475    | 1.687 ± 0.530  | 2.22 ± 0.728           |

According to the analysis of the nucleotide composition of the COI gene sequences, there are differences in the composition of *Barbodes binotatus*. This shows that there are genetic variations of *B. binotatus*. The results of the analysis of the nucleotide content of the entire population showed that guanine-cytosine (G: C) was lower than adenine-thymine (A: T) (Table 4). Hydrogen bonds are known as weak bonds when compared to covalent bonds, so that the C-G basic bonds have a lower resistance than the A-T base bonds. The nature of the weak hydrogen bond causes the bond to break easily and changes in the base molecular structure of DNA [20] [21].
Table 4. Nucleotide Base Components of the Sample

| No. | Location  | C   | T   | A   | G   | A+T | C+G   |
|-----|-----------|-----|-----|-----|-----|-----|-------|
| 1   | Pasuruan  | 27.18 | 29.31 | 26.72 | 16.79 | 50.03 | 43.97 |
| 2   | Yogyakarta | 27.33 | 29.16 | 26.56 | 16.95 | 55.72 | 44.28 |
| 3   | Cirebon   | 27.33 | 29.16 | 26.41 | 17.10 | 55.57 | 44.43 |

The results showed that populations in Pasuruan, Yogyakarta and Cirebon (Table 5) have low genetic diversity (the number of haplotypes is one, haplotype diversity (Hd) 0.000 and nucleotide diversity (\(\Pi\)) 0.000). Endemic species generally have low genetic diversity values. Species that live in isolated environments also generally have low genetic diversity due to increased deletion processes that occur due to inbreeding activity and the occurrence of genetic drift or gene drift in small populations [22].

Table 5. Genetic Diversity of the Sample

| No. | Location  | N | Base Pair | H | Hd | \(\Pi\) |
|-----|-----------|---|-----------|---|----|-------|
| 1   | Pasuruan  | 9 | 655       | 1 | 0.000 | 0.00000 |
| 2   | Yogyakarta | 8 | 655       | 1 | 0.000 | 0.00000 |
| 3   | Cirebon   | 10 | 655      | 1 | 0.000 | 0.00000 |

Genetic drift or gene drift is a change in the genetic composition of a population due to random sampling in a small population. This activity gives an impact for genetic diversity, random changes in allele frequencies and diversification in other populations [23]. The lowest genetic diversity of some populations also caused by migration rates and isolated areas. This also causes inbreeding to become larger.

Table 6. Number of Haplotype

| No. | Haplotype | N | Keterangan |
|-----|-----------|---|------------|
| 1   | Hap_1     | 9 | JTMA1, JTMA2, JTMA3, JTMA4, JTMA5, JTMA6, JTMA7, JTMA8, JTMA9 |
| 2   | Hap_2     | 7 | JTYB1, JTYB2, JTYB3, JTYB4, JTYB5, JTYB6, JTYB7, JTYB8 |
| 3   | Hap_3     | 10 | JBRC1, JBRC2, JBRC3, JBRC4, JBRC5, JBRC6, JBRC7, JBRC8, JBRC9, JBRC10 |

The lowest value of genetic diversity in a species in the long term will cause several negative impacts, such as decreased genetic variation and higher homozygotes, so that the genetic diversity of species living in isolated populations tends to be small due to low gene flow and small population size [24] [25]. This activity can cause a decrease in the level of fitness and survival due to environmental changes, and it is necessary to cross between populations through hatchery and domestication [26]. Some populations that have a high value of genetic diversity generally have a high level of adaptability due to genotypic variations that appear in response to changes in environmental conditions [27]. The high genetic diversity causes more genes to be involved in the ability of a population to survive and adapt to the environment in which it lives [28]. The results of phylogenetic analysis were carried out using MEGA software. Phylogenetic tree construction is one of the most frequently used methods to understand the kinship of living thin, which is analyzed through phylogenetic relationship reconstruction. Phylogenetic tree construction was carried out using main data (population samples of Pasuruan, Yogyakarta and Cirebon) with comparable data from 13 population areas of \(B.\ binotatus\) (Appendix 10). The species outgroups selected in the phylogenetic analysis were \(Barbonymus\ altus\) (BNMAL01), \(Barbonymus\ gonionotus\) (BNMG02), and \(Barbonymus\ balleroides\) (BMBAL01). Based on the results of phylogenetic analysis, it was found that the wader cakul (\(B.\ binotatus\)) sample from the Tawang River (Pasuruan) has close relatives with \(B.\ binotatus\) from the Kediri and Mojokerto regions.
According to a geographical perspective, the Pasuruan, Kediri and Mojokerto areas are the watershed of the Brantas River. This illustrates that the cakul wader population with close kinship values in the Pasuruan, Kediri and Mojokerto areas may still be influenced by the same geographical conditions and river flow. The same thing also resulted from the population in the area of the Opak River (Yogyakarta) and the Samin River (Karanganyar, Central Java) which showed a close relationship. Samples of *B. binotatus* from the Cisanggarung River (Cirebon) showed the proximity of the phylogenetic tree to *B. binotatus* from Kali Timbang (Tegal) and Cijuray (Purwakarta). Another result shows that *B. binotatus* from Buleleng (Bali) shows closeness to the *B. binotatus* population in Lumajang.

The highest paired F$_{ST}$ coefficient value is obtained in the combination of Pasuruan - Yogyakarta, Pasuruan - Cirebon, Yogyakarta - Cirebon, Pasuruan - Tegal, Pasuruan - Temanggung, Pasuruan - Bali, Yogyakarta - Mojokerto, Yogyakarta - Purwakarta, Yogyakarta - Tegal, Cirebon - Karanganyar, Cirebon - Kediri, Cirebon - Temanggung and Cirebon - Bali with a coefficient value of 1.00 (F$_{ST}$ > 0.25). This indicates that there is a process of genetic differentiation so that there is no gene flow between the two locations. Genetic differentiation with low values is shown in the combination of Bali - Lumajang, Tegal - Purwakarta, Yogyakarta - Temanggung with a value of 0.000 (F$_{ST}$ = 0.00 - 0.5). The lowest F$_{ST}$ value indicates that there is no genetic differentiation in this population. Genetic flow rates with a value of less than 10 can limit the flow of genes, causing genetic subdivisions. In particular, freshwater species
show a higher level of genetic population differentiation from seawater species (anadromes). This occurs because of the potential for migration barriers and the presence of less gene flow [29]. In fact, some cases have been shown that the movement of species between areas does not necessarily lead to gene flow. The absence of gene flow will give a greater possibility of genetic diversity between species. Genetic variation is very important as one of the things related to the adaptability of a population [30]. The loss of genetic variation due to inbreeding or isolation will ultimately result in decreased adaptation potential and population’s ability to survive [31].

![Figure 4](image)

**Figure 4.** The results of the F_{ST} analysis (p<0.05 = different (*)).

| Location Description | 1) Pasuruan, 2) Yogyakarta, 3) Cirebon, 4) Pandeglang, 5) Lumajang, 6) Ambarawa, 7) Karanganyar, 8) Jepara, 9) Mojokerto, 10) Tegal, 11) Kediri, 12) Purwakarta, 13) Temanggung, 14) Bali, 15) Tasikmalaya, 16) Sukabumi.

4. **Conclusion**

Genetic diversity of the Tawang River (Pasuruan), Opak River (Yogyakarta) and Cisanggarung River (Bogor) is low with the number of haplotypes (h) one (h=1), haplotype diversity (Hd) = 0.000 and nucleotide diversity (Π) = 0.000. The population structure analysis showed that there was genetic differentiation between populations of Pasuruan, Yogyakarta and Cirebon with an F_{ST} value of 1.00.

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