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## RESEARCH ARTICLE

# MITOCHONDRIAL DNA MARKER-BASED IDENTIFICATION AND GENETIC DIVERSITY OF RABBITFISH (*SIGANUS* SPP)

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## ABSTRACT

This study was aimed at determining the identity and the genetic diversity of rabbitfish based upon mtDNAmolecular marker. Rabbitfish specimens were collected from Bahu waters (n=17), Manado Bay. DNA was extracted using 10% chelexsolution. FF2d andFR1d primer pairs were used for CO1 gene fragment amplification. PCR applied 30 cycles, and the output was sequenced, blasted, and analyzed using various softwares to achieve the objectives. Results found a total of 8 species,*Siganusargenteus, S. virgatus, S. vulpinus, S. doliatus, S. luridus,S. puellus, S. punctatus,* and *S. punctatissimus.* Genetic diversity of Siganid was high, and 13 haplotypes were identified from all sequences. Haplotypeand nucleotide diversity reached 0.97 and 0.06, respectively. Each species possessed different haplotypes. *S. vulpinus, S. virgatus, S. luridus, S. doliatus,* and *S. argenteus* had 100% haplotype frequency. *S. punctatus* was represented by 6 individuals with the haplotype frequency higher than 66%, *S. puellus* had 50% frequency, and *S. Punctatissimus* had 100%.



## INTRODUCTION

Rabbitfish are a typical fish group with particular characteristics of longitudinal and laterally flat body, covered with small scales, and have terminal small mouth. Jaw is facilitated with small teeth. Dorsal part is armored with sharp strong spines containing toxin at the anal and the dorsal fins (Johnson and Gill, 1998).Rabbitfish belong to Siganidae. This familiy comprises one genus (*Siganus*) and 2 sub genera, *Siganus* and *Lo* (D.J. Woodland 1990;J.E. Randall, 2005). Rabbitfish occur in sufficient variations and can be easily identified at the daytime. In the night or when threatened, their color can change (faded), anddark spots drastically appear. When they die, the color will also quickly fade. They have universal meristic characters as well, and their identification is particularly based on the color character. This color change makes the species difficult to recognize along their life history, so that morphological identification could be difficult to doin siganid (Kuiter 1993; Thresher 1984). Accurate species identification is the most fundamental thing in aquatic resources management effort. Cytochrome Oxydase 1 (CO1) gene fragment molecular analysis-based species identification, called DNA Barcoding, is one of the general standard methods recently applied. The advantage of the method is its ability to identify morphologically indistinguishable species (Hebert *et al.*, 2004), useanimal samples of all life cycles including eggs and larvae (Stoeckle, 2003),and utilize very few tissues so that there is no need to kill the animal. Nevertheless, this method is highly dependent upon the availability of accuratereference sequence data as comparison (Stoeckle, 2003). Other advantages are its ability to provide genetic information for broader scientific usages, such as phylogenetics (Erickson and Driskell, 2012; Huang *et al*. 2016), phylogeography (Yu 2014), andpopulation genetics (Draft *et al*. 2010).Meanwhile, genetic variationhas crucial meaning in population stability and sustainability (Ferguson *et al*. 1995), and genetic diversity has direct and indirect impact on population, community, and ecosystem (Hughes *et al*. 2008). Loss in genetic diversity reduces species ability to adapt to environmentalchanges (Frakham, 1999). Information on genetic diversity patterns could also become centre of future efforts in order for species conservation (IUCN 2007; http://www.iucnredlist.org) and has conservational and management implications (Ning*et al*. 2015). DNA barcoding application and genetic variation study of rabbitfish have not been done in Indonesia in general andNorth Sulawesi in particular. It results in low number of CO1 gene DNA sequences of the rabbitfish from Indonesia recorded in the gen

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bank (www.ncbi.nlm.gov/). This study was aimed to identify the rabbitfish species from Bahu waters-Manado Bay, North Sulawesi, based on CO1 gene fragment DNA sequence and to know their genetic variations. The former is expected to become supporting data for rabbitfish resources management in Indonesia, especially North Sulawesi, and could augment the genbank data of rabbitfish DNA sequences.The latter is also expected to be able to determine the life status of the population on study.

## METHODS

### Sample Collection

Fish samples were collected from Bahuwaters, ManadoBay (Fig. 1). Seventeen specimens were taken for genetic analysis. The body tissue of dorsal near caudal peduncle was removed and put into a 95% alcohol-containing jar, brought to the laboratory, and stored until usage.

**Fig. 1. Map of sampling site in Bahu waters, Manado Bay-North Sulawesi**

### DNA Extraction, PCR, and Sequencing

Rabbitfish DNA extraction was carried out using 10%chelexsolution (Walsh *et al*. 1991). Tissue cut of the specimen was put into chelex-containing flask and heated in aheating blockat 90$^{o}$C for 25 min., vortexed and centrifuged, respectively, for 30 seconds. DNA genomewas obtained assupernatant.

CO1 gene fragment was amplified from thesupernatantusing primer pairs, forward FF2d: 5'-TTCTCCACCAACCACAARGAYATYGG-3' and reverse FR1d:5'-CACCTCAGGGTGTCCGAARAAYCARAA-3' (N.V. Ivanova*et al*. 2007). The composition for PCR process (25µl) was as follows: 2.5µl of dNTPs(8 µM), 2.5 µl of PCR Buffer (10x), 1.25 µl of forward primer (10 mM), 1.25 µl of reverse primer (10 mM), 2 µl of MgCl2 (25 mM), 0.125 µl of Amplitag (5 unit/ l), 2 µl of DNA genom, and 14.5 µl dd$H_2O$. Amplification was done in a Thermal cycler Biorad 48-Well machine. PCR reaction was run as follows: initial denaturation for 30 cycles at 94$^{o}$C (5 min.),denaturation at 94$^{o}$C (30 sec.), primer attachment at 50$^{o}$C (30 sec.), elongation at 72$^{o}$C (45 sec.), andfinal elongation at 72 $^{o}$C(10 min.). The PCR outcomes were sequenced using commercial laboratory service in Malaysia to obtain the sequence data of the rabbitfish.

### Data Analysis

The sequence was aligned usingClustalWof version 5-2-MEGA software (Tamura *et al*., 2011).The aligned sequences were then exported to the NCBI for species identity analysis using Basic Local Alignment Search Tool (BLAST) program. The outcome of DNA matching to the highest homologous value of the first sequence number was expressed as sample identity. Futher identification was done using Disparity Index Test of Substitution Pattern Homogeneity (Kumar and Gadagkar 2001) or intersequential disparity test. Monte Carlo (1000 replications)was also applied to estimate P-value (Kumar and Gadagkar 2001). The analysis employed version 5.2-MEGA (Tamura *et al*. 2011).The next steps were to measure the genetic distance of each individual and build the phylogenetic tree to determine the species identity. Genetic distance differences between taxa were calculated based upon p-distance model. The kindship between taxa was analyzed based on neighbor-joining (NJ) method andreconstructed through 1,000 replications (*boostrap*). Genetic diversity was analyzed based on CO1 sequence base composition variable using version 5.2-MEGA software (Tamura *et al*., 2011), while the haplotype variables, haplotype diversity and nucleotide diversity used version 5.10.01-DnaSP. (Rozas *et al*. 2010).

## RESULTS

Nucleotide sequence data of sequencing process found 538bp (base pairs). All nucleotides were taken to determine the species identity and the genetic diversity of the rabbitfish (Siganid).

### Genetic Identity

BLAST outcomes of all nucleotide sequences (636bp)of the rabbitfish succeeded to identify sequence similarity of various species.Fourteen sequences identified more than one species (species identity of 94-100%), while 3 others identified a single species (speciesidentity of 99-100%). All sequences were grouped in 8 species. Further analysis on disparity index and genetic distance supports these results (Table 1). Table1 demonstrates that the identity of 98-100%, zero disparity index, and very low genetic distance up to 0.00 reflect high similarity between the studied sequence and the genbank sequence. The complete matrix of entire sample genetic distance is presented in Table 3. Phylogenetic analysis also supports the result above that all sequences belong to 8 species(Fig. 2). All sequences on study belong to the same cluster as the genbank reference and possess similarity value up to 100%. It reveals that all sequence samples consist of 8 samples of *S. argenteus*, *S. virgatus,S. vulpinus*, *S. doliatus, S. luridus*, *S. puellus, S. punctatus*, and*S. punctatissimus*.

### Genetic Variation

Transition/transverse (*R*) bias estimation of the 8 species was 4.88. Nucleotide frequency A = 26.43%, T = 26.43%, C = 23.57%, and G = 23.57%. all sequences consisted of 430bpof

**Table 1. Identity of sequence on study based upon comparison with genbank sequence through BLAST analysis, disparity index test, and genetic distance test on CO1 gene sequences**

| No. | Sample Code | Species of BLAST outcome | *Identity value* (%) | Access Codeof NCBI*) | Disparity index.*P***) | Genetic distance |
|---|---|---|---|---|---|---|
| 1 | SIG46 | *S. argenteus* | 100 | gi\|628820497\|KJ202205.1 | 0.00; 1.00 | 0.00 |
| 2 | SIG107 | *S. virgatus* | 100 | gi\|223369142\|FJ584112.1 | 0.00; 1.00 | 0.00 |
| 3 | SIG117 | *S. vulpinus* | 100 | gi\|223369148\|FJ584115.1 | 0.00; 1.00 | 0.00 |
| 4 | SIG106 | *S. doliatus* | 100 | gi\|584296893\|KF930440.1 | 0.00; 1.00 | 0.00 |
| 5 | SIG108 | *S. luridus* | 98 | gi\|952025488\|KR905701.1 | 0.00; 1.00 | 0.02 |
| 6 | SIG9 | *S. puellus* | 100 | gi\|223369134\|FJ584108.1 | 0.00; 1.00 | 0.00 |
| 7 | SIG11 | *S. puellus* | 100 | gi\|223369134\|FJ584108.1 | 0.00; 1.00 | 0.00 |
| 8 | SIG42 | *S. puellus* | 99 | gi\|223369134\|FJ584108.1 | 0.00; 1.00 | 0.00 |
| 9 | SIG99 | *S. puellus* | 99 | gi\|223369134\|FJ584108.1 | 0.00; 1.00 | 0.00 |
| 10 | SIG8 | *S. punctatus* | 99 | gi\|816375897\|KP194265.1 | 0.00; 1.00 | 0.00 |
| 11 | SIG41 | *S. punctatus* | 99 | gi\|816375897\|KP194265.1 | 0.00; 1.00 | 0.00 |
| 12 | SIG59 | *S. punctatus* | 99 | gi\|816375897\|KP194265.1 | 0.00; 1.00 | 0.00 |
| 13 | SIG77 | *S. punctatus* | 100 | gi\|339431776\|JF952857.1 | 0,00; 1,00 | 0.00 |
| 14 | SIG78 | *S. punctatus* | 100 | gi\|816375897\|KP194265.1 | 0.00; 1.00 | 0.00 |
| 15 | SIG116 | *S. punctatus* | 100 | gi\|816375897\|KP194265.1 | 0.00; 1.00 | 0.00 |
| 16 | SIG114 | *S. punctatissimus* | 99 | gi\|816376705\|KP194669.1 | 0.00; 1.00 | 0.00 |
| 17 | SIG100 | *S. punctatissimus* | 99 | gi\|816376705\|KP194669.1 | 0.00; 1.00 | 0.00 |

Note: *) access code for other sequence is presented in Fig. 2; **) Null hypothesis (*Ho*): not different, *Hi:* different;reject *Ho* if*P*<0.05 at 95% confidence level.

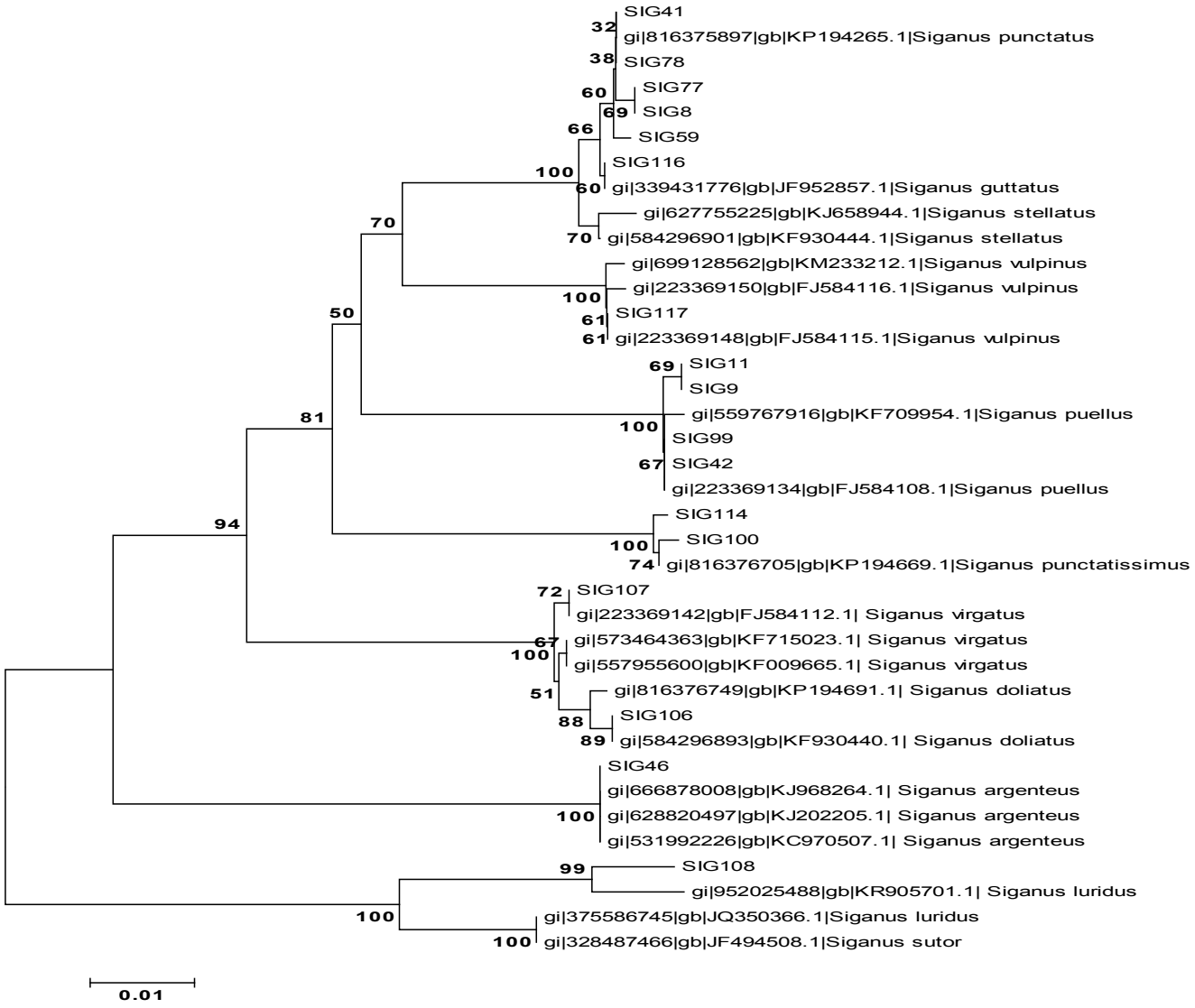


**Fig. 2. Phylogenetic Tree.Analysis on 17 rabbitfish sequences (SIG code) combined with genebank sequence possessing high identity based on BLAST analysis (code under species names, for instance,*Siganusluridus*, *S. Sutor*, andetc.). It is seen that the study sequences refer to the genbank species in line with their evolutionary history**

**Table 2. Haplotype composition of each species**

| Species | No.Individuals | Haplotype | |
|---|---|---|---|
| | | Number | Type (ind.) |
| *S. argenteus* | 1 | 1 | Hap_1 |
| *S. doliatus* | 1 | 1 | Hap_2 |
| *S. luridus* | 1 | 1 | Hap_3 |
| *S. puellus* | 4 | 2 | Hap_4 (2 ind), Hap_5 (2 ind) |
| *S. punctatissimus* | 2 | 2 | Hap_6 (1 ind), Hap_7 (1 ind) |
| *S. punctatus* | 6 | 4 | Hap_8 (2 ind), Hap_9 (2 ind), Hap_10 (1 ind), Hap_11 (1 ind) |
| *S. virgatus* | 1 | 1 | Hap_12 |
| *S. vulpinus* | 1 | 1 | Hap_13 |
| | 17 | 13 | |

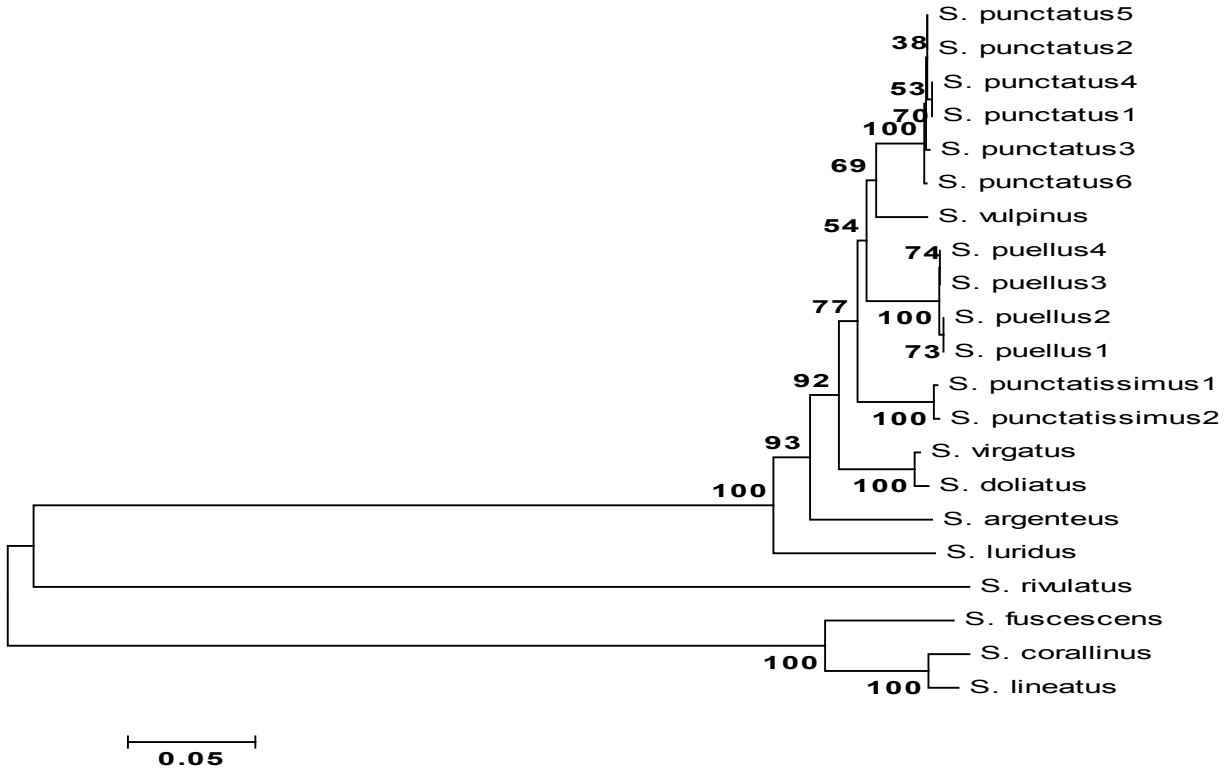


**Fig. 3. Phylogenetic tree of 8 species. A totalof 17 species analyzedwas added with 4 genbank sequences asoutgroup. *S. corallinus* (gi|816377109|gb|KP194871.1|), *S. lineatus*(gi|816376815|gb|KP194724.1|), *S. rivulatus* (gi|752295367|gb|KM538561.1|), and*S. fuscescens* (gi|592747553|gb|KJ013061.1|) is 4 speciesin genus Siganusasout group**

**Table 3. Interspecific genetic distance**

| Spesies | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *S._argenteus*(1) | | | | | | | | | | | | | | | | | |
| *S._doliatus*(2) | 0.09 | | | | | | | | | | | | | | | | |
| *S._luridus*(3) | 0.12 | 0.12 | | | | | | | | | | | | | | | |
| *S._puellus1* (4) | 0.11 | 0.08 | 0.13 | | | | | | | | | | | | | | |
| *S._puellus2* (5) | 0.11 | 0.08 | 0.13 | 0.00 | | | | | | | | | | | | | |
| *S._puellus3* (6) | 0.11 | 0.08 | 0.13 | 0.00 | 0.00 | | | | | | | | | | | | |
| *S._puellus4* (7) | 0.11 | 0.08 | 0.13 | 0.00 | 0.00 | 0.00 | | | | | | | | | | | |
| *S._punctatissimus1* (8) | 0.11 | 0.07 | 0.13 | 0.06 | 0.06 | 0.06 | 0.06 | | | | | | | | | | |
| *S._punctatissimus2* (9) | 0.11 | 0.07 | 0.13 | 0.06 | 0.06 | 0.06 | 0.06 | 0.00 | | | | | | | | | |
| *S._punctatus1* (10) | 0.09 | 0.07 | 0.12 | 0.06 | 0.06 | 0.05 | 0.05 | 0.06 | 0.06 | | | | | | | | |
| *S._punctatus2* (11) | 0.09 | 0.07 | 0.12 | 0.05 | 0.05 | 0.05 | 0.05 | 0.06 | 0.06 | 0.00 | | | | | | | |
| *S._punctatus3* (12) | 0.09 | 0.07 | 0.12 | 0.06 | 0.06 | 0.05 | 0.05 | 0.06 | 0.06 | 0.00 | 0.00 | | | | | | |
| *S._punctatus4* (13) | 0.09 | 0.07 | 0.12 | 0.06 | 0.06 | 0.05 | 0.05 | 0.06 | 0.06 | 0.00 | 0.00 | 0.00 | | | | | |
| *S._punctatus5* (14) | 0.09 | 0.07 | 0.12 | 0.05 | 0.05 | 0.05 | 0.05 | 0.06 | 0.06 | 0.00 | 0.00 | 0.00 | 0.00 | | | | |
| *S._punctatus6* (15) | 0.09 | 0.07 | 0.12 | 0.06 | 0.06 | 0.05 | 0.05 | 0.06 | 0.06 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | | | |
| *S._virgatus*(16) | 0.09 | 0.01 | 0.12 | 0.08 | 0.08 | 0.08 | 0.08 | 0.06 | 0.07 | 0.07 | 0.07 | 0.07 | 0.07 | 0.07 | 0.07 | | |
| *S._vulpinus*(17) | 0.10 | 0.07 | 0.13 | 0.05 | 0.05 | 0.05 | 0.05 | 0.06 | 0.06 | 0.04 | 0.04 | 0.04 | 0.04 | 0.04 | 0.04 | 0.06 | |

monomorphic DNA and 108bpof olymorphic DNA. The latter came from 48bpof singleton variables and 60bpof informative parsimony. Total number of mutations was 132bp. Haplotype and nucleotide diversity was entirely 0.97 and 0.06, respectively. This study identified 13 haplotypes. The haplotype of each species is presented in Table 2. Each species has different haplotype. Species represented by one sequence or individual (*S. vulpinus, S. virgatus, S. luridus, S. doliatus, and S. argenteus*) have one haplotype (haplotype frequency of 100%). Similar situation occurs in inter-individual genetic diversity in one species. *S. punctatus* standing for 6 individuals has haplotype frequency of more than 66%, *S. puellus* has 50%, and*S. Punctatissimus* has 100%. Genetic kinship of 8 species (17 individuals) is presented in Fig. 3.

The same species represented by more than one individual joins one cluster indicating the same origin or evolutionary species. Species standing for one individual forms separate cluster from other species.*S. doliatus* and *S. virgatus* seems to be in one cluster due to closely related, while *S. vulpinus* belongs tothe same cluster as*S. Punctatus* due to their evolutionary closeness. It reflects similarities and differences of each species. Four out group species make discrete cluster from those in this study.

CO1 gene sequence-based genetic distance matrix of 8Siganusspecies is presented in Table 3. Interspecific genetic distance in genus Siganusranges from 0.01 to 0.13, while inter-individual genetic distance of one species, such as *S. puellus*, *S. punctatissimus*, and *S. punctatus,* is 0.000.

## DISCUSSION

This study identified 8 species of rabbitfish belonging to Siganid, *Siganusargenteus*, *S. doliatus, S. luridus, S. puellus, S. punctatissimus, S. punctatus, S. virgatus,*and *S. vulpinus*. Siganidae comprised of 28 species based uponmorphology and color patterns (Woodland, 1990; Randall and Kulbicki, 2005). Therefore, this study has identified up to 28.57% of siganid species worldwide. Based on genetic identification on the entire samples, it is apparent that there is difference in species composition with study site. The most dominant species in this study was *S. punctatus* (35%), followed by *S. puellius* (23%) and *S. punctatissimus* (12%). Five other species were*S. argenteus*, *S. virgatus*, *S. vulpinus*, *S. doliatus*, and *S. luridus* (6% of each).

This high species composition could result from spawning stock abundance and high species diversity in Manado waters. Other waters contains different species composition. For instance,different number of species was found in Spermonde waters, 13 species (M. Yunus, 2005) and 16 species (BurhanuddinandIwatsuki, 2006), respectively, while the rabbitfish caught in Banten waters were *S. canaliculatus*, *S. guttatus*, *S. virgatus*, *S. javus*, *S. chrysospilos* and *S. vermiculatus* (Nurhakim, 1984)

The eight species of this finding are different from those reported by other experts. According toFisheries Directorate General (2001), siganids in Indonesia waters were*Siganusguttatus*, *S. canaliculatus*, *S. vulpinus*, *S. virgatus*, *S. corallinus*, *S. spinus*, *S. puellus*, *S. javus*, *S. lineatus*, *S. doliatus*, *S. chrysospilos*, and *S. fuscencens.*Indonesian rabbitfish were identified as*Siganusguttatus*,*S. canaliculatus*, *S. vulpinus*, *S. virgatus*, *S. corallinus*, *S. spinus*, *S. vermiculatus*, *S. puellus*, *S. javus*, *S. punctatus*, *S. argenteus*, and *S. Fuscencens* (Carpenter, 2001)**.** Other study in North Sulawesi waters recorded 13 siganid species,*Siganusargenteus*, *S. canaliculatus, S. coralinus, S. doliatus, S. fuscescens, S. gutatus, S. lineatus, S. puellus, S. punctatissimus, S. punctatus, S. stellatus, S. vermiculatus,* and *S. Vulpinus* (Makatipu *et al*. 2010). Thus,this study found 3 species identical to those from identified by Directorate General of Fisheries (2001), *S. vulpinus*, *S. virgatus*, and*S. puellus,* 5 species to Carpenter (2001), *S. vulpinus*, *S. virgatus, S. puellus*, *S. punctatus*, and *S. argenteus,* and6 speciesto Makatipu *et al*. (2010), *S. argenteus*, *S. doliatus*, *S. puellus, S. punctatissimus, S. punctatus*, and*S. vulpinus*.

This difference could probably be related with different approach or identification method used, such as.morphology and color patterns (Woodland 1990) and allozymes (Lacson and Nelson, 1993) in Siganid identification. This study utilized molecular approach using CO1 sequence of all samples for species identification. The accuracy of barcoding method was highly dependent upon the availability of accurate reference sequence dataas comparison (Stoeckle, 2003). Moreover, genetic diversity of siganid from North Sulawesi waters is very high. It was indicated with number of haplotypes and their haplotype diversity. High genetic diversity of siganid was also recorded in Red Seawith 21 haplotypes (26 samples)andhaplotypediversity of 0.978, and 21 haplotypes in Mediteranian Sea with haplotype diversity of 0.88 (Azzurro *et al*., 2006). This study shows lower nucleotide diversity than that of siganid in the Red Sea andMediteranian Sea, 0.95 and 0.59 nucleotide diversity, respectively. All siganid species and interspecific siganid in this study have very close kinship (Fig.3 and Table 3). One species of several individuals forms the same cluster reflecting that the individual species possesses similar evolutionary history. On the other hand, more than one species in single cluster reveal interspecific kinship. *Siganusdoliatus* and *S. Virgatus* seemed to be one cluster due to considered as closely related species (P. Borsa*et al*. 2007) andbelieved as hybrid (J.E. Randall 2005). *S. vulpinus*is in the same cluster as *S. punctatus* because of having an evolutionary closeness. In addition, molecular phylogeny could provide information on species biogeographic hypothesis and testable ecological speciation scenario (Borsa*et al*. 2007).

### Conclusion

Based on genetic analysis, it was found that the fish samples consisted of 8 species, *S. argenteus* (6%), *S. virgatus* (6%),*S. vulpinus* (6%), *S. doliatus* (6%), *S. luridus* (6%), *S. puellus* (23%), *S. punctatus* (35%), and *S. punctatissimus* (12%), with very high genetic variations. This study also discovered 13 haplotypes with haplotype diversity of 0.97 andnucleotide diversity of 0.06. All Siganid species from North Sulawesi waters possessed very close kindship.

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