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Abstract. The Asian seabass (*Lates calcarifer*) is an important marine food fish in Southeast Asia. To facilitate a selective breeding program conducted in Indonesia, we genotyped 29 Asian seabass specimens collected from four wild populations in the Wallacea Region originating from coastal waters in two seaways: the Makassar Strait (Bulungan in North Kalimantan and Maros in South Sulawesi) and the Gulf of Bone (Siwa and Bone in South Sulawesi). We used four polymorphic markers with a random amplified polymorphic DNA (RAPD) method. A total of 43 alleles were detected at four loci across the four populations, four of which were shared by all the accessions while 39 were polymorphic. Of these 43 alleles, 34.88% were unique, with 46.67% in the Gulf of Bone and 53.33% in the Makassar Strait. Asian seabass within-population genetic distance was greater in Bone Bay than the Makassar Strait, and an ongoing speciation phenomenon is suspected. The greatest genetic distance (0.291) was between the Asian seabass populations from Bulungan, North Kalimantan and Cenranae River estuary, Bone Regency; the lowest (0.086) was between the Bulungan and Maros populations. In the phylogenetic analysis, Gulf of Bone and Makassar Strait specimens formed separate clades. Commercial scale cross-breeding between Asian seabass from Makassar Strait and the Gulf of Bone may produce highly adaptable seed. These results will be helpful in future Asian seabass breeding programs as well as for optimising management and conservation strategies for wild Asian seabass populations.

1. Introduction

Asian seabass (*Lates calcarifer* Bloch, 1790) is a widely cultivated fish species in Southeast Asia, Taiwan, China, Hong Kong, India, Saudi Arabia, and Australia [1–4]. In Australia this fish is known as barramundi for marketing reasons. As a farmed fish, the Asian seabass has several advantages such as a high growth rate, reaching 3-5 kg in 2-3 years, the ability to adapt to high stocking density, and as a euryhaline species it can be kept in seawater, brackish water or freshwater. With wide market appeal,

this high-value fish sells for US $ 12-13/kg. Asia seabass aquaculture began in Thailand in the early 1970s and then quickly spread to other Southeast Asian countries [1] and Australia [5]. The main Asia seabass producing countries are Thailand, Malaysia, Taiwan, Indonesia, Singapore, and Australia [6], with a global production of 71,581 tonnes in 2014 rising to 90,000 tonnes by 2017 [7]. Asian seabass production in Indonesia is being further increased through growout technologies including offshore marine cages, pond culture, and fish-rice polyculture. [8].

Constraints on Asian seabass production in Indonesia include limited seed production and poor seed quality. This is presumed to be due to the low number of broodstock in hatcheries, however genetic diversity has not been controlled. One way to increase aquaculture production can to optimize the use of wild populations by exploring the potential for different strains from several regions and the application of molecular markers to facilitate selection and control for genetic diversity. Singapore began implementing a selective breeding program in 2003 using microsatellite markers [9], followed by Malaysia in 2005 and India in 2012 random amplified polymorphic DNA (RAPD) markers [3,10].

Genetic diversity is the basis of biodiversity; therefore it is a valuable asset and must be preserved. There are several reasons for maintaining genetic variation: (1) genetic diversity is required for populations to adapt traits of importance as a response to environmental change/natural selection; (2) genetic variation provides important information for conservation, ecological and systematic studies and evolution; (3) genetic diversity tends to be positively related to characteristics which are desirable in aquaculture [11–13]. Therefore, since 1990 the World Conservation Union (IUCN) has included genetic diversity as one of the three forms of biodiversity that must be conserved.

To increase Asian seabass seed production volume and quality in Indonesia, new superior broodstock with high genetic diversity are urgently needed. Broodstock with high genetic diversity can be obtained by hybridization of individuals from several different natural and cultivated parent stock sources. With respect to this matter, in 2018 Irmawati et al. [8] began mapping the distribution and genotypes of Asian seabass stocks in Indonesia. Tracking, analysis, monitoring and evaluation of the genetic diversity of the Asian seabass wild stock and cultured stock is important to elicidate the potential of Asian seabass germplasm in Indonesia populations as a source of ecosystem biodiversity and as a basis for producing superior broodstock. The availability of such data and improved understanding of genetic information will greatly assist in the efficient and wise utilization of Asian seabass resources so as not to reduce genetic diversity.

Although data on the genetic diversity of Asian seabass in Western Indonesia (specifically in the waters of South Sumatra and Papua) have been reported by[14], information is lacking on the genetic diversity of Asian seabass in other areas in Indonesia, in particular the Wallacea region. Therefore, this study aimed to analyze the genetic diversity of Asian seabass in four populations (germplasm sources) in the Wallacea region, with two sites in the Makassar Strait (Bulungan, North Kalimantan and the Kuricad River estuary in Maros Regency, South Sulawesi) and two sites in the Gulf of Bone (Siwa in Wako Regency and the Cenranae River estuary in Bone Regency, South Sulawesi) using the random amplified polymorphic DNA (RAPD) method.

2. Materials and methods

2.1. Asian seabass sample collection

Asian seabass samples were obtained from five specimens collected in the Kuricad River estuary in Maros Regency, South Sulawesi, 10 specimens from the coastal waters of Bulungan Regency, North Kalimantan, and seven specimens each from the estuary of the Cenranae River, Bone Regency and the coastal waters of Siwa in Wajo Regency, both in South Sulawesi (Figure 1).

Figure 1. Sampling localities (shown as fish symbols, with number of specimens) of four Asian seabass populations in the Wallacea Region from Bulungan in North Kalimantan to the Gulf of Bone

2.2. Extraction and quantification of Asian seabass genomic DNA

Genomic DNA of the Asian seabass was extracted using the *DNeasy Blood & Tissue Kit* (QIAGEN, Germany) following the manufacturer's protocol. Total genomic DNA was analysed using the Qubit *ds*DNA BR (broad range) Assay Kit (Invitrogen, USA), also following manufacturer's protocols.

2.3. PCR-RAPD (*Polymerase Chain Reaction-Random Amplified Polymorphic DNA*)

The first stage in the PCR-RAPD analysis was to select primers and determine the optimum temperature for each primer. The primers resulting in polymorphic electrophoresis bands were chosen (Table 1).

Table 1. Oligonucleotide primers and annealing temperatures used to analyse the genetic diversity of Asian seabass (*Lates calcarifer* Bloch, 1790) populations

Primer	Nucleotide sequences	Temperature (^{o}C)
OPA-02	5'-TGC CGA GTC G-3'	40.7
OPA-15	5'-TTC CGA ACC C-3'	34.2
OPD-20	5'-ACC CGG TCA C-3'	39.1
OPP-08	5'-ACC CGG TCA C-3'	36.0

Each PCR-RAPD reaction comprised 3 µL nuclease free water, 6.25 µL KAPPA PCR mix, 1.25 µL primer, and 3 µL DNA giving a total PCR mix volume of 13.5 µL. The PCR amplification parameters were: initial denaturation at 95^{0} C for three minutes followed by 35 cycles with denaturation at 95^{0} C for 30 seconds, annealing at 39.1^{0} C (depending on the primer) for 50 seconds and extension at 72^{0} C for one minute. The DNA amplification process finished with a final extension

at 72^0 C for 5 minutes. The amplified PCR product was then stored at 4^0 C for an indefinite period until used in the following stages of the analysis.

2.4. Electrophoresis

The PCR-RAPD extracted and amplified DNA product was placed in wells in 2% agarose gel plaques placed in electrophoresis buffer. A 100 *bp* DNA ladder (Invitrogen) was also placed in one well. The electrophoresis was run at 100 volts for 90 minutes. The DNA bands were visualised under an ultra-violet (UV) transluminator. The bright bands represented segments of DNA which had been amplified. Differences in the patterns of the bands produced reflect the genetic variation between the Asian seabass specimens sampled.

2.5. Data analysis

The first stage in the data analysis was to score the DNA bands based on the nucleotide fragment lengths using the 100 *bp* DNA ladder (Invitrogen). The DNA bands were converted to binary data based on nucleotide length and analysed in Darwin 6.0 ref to determine the genetic distance within and between populations, identify polymorphisms, and build a phylogeny of the specimens. The phylogenetic analysis used the Neighbor-joining routine with 500 bootstrap repeats.

3. Results and discussion

The results revealed relatively high allelic richness, with 12-2 alleles per locus and 93.85% polymorphic alleles. Of a total of 43 alleles found at four loci, 15 (34.88%) were unique alleles, 7 from the Gulf of Bone Asian seabass population and 8 from the Makassar Strait population (Table 2).

Table 2. Asian seabass allelic richness and unique alleles

Primer	Allele	Monomorphic	Polymorphic	Total	Unique alleles	
					Molecular Weight (bp)	Sites
OPD-20	20	0	20	4	95	Cenranae River estuary
					105	
					340	Siwa coastal waters & estuary of Cenranae River
					1300	Kuricaddi River estuary, Maros & Bulungan coastal waters
OPP-08	15	0	15	2	550	Kuricaddi River estuary, Maros & Bulungan coastal waters
					1000	Kuricaddi River estuary, Maros
OPA-15	12	0	12	5	280	Kuricaddi River estuary, Maros & Bulungan coastal waters
					700	
					750	Bulungan coastal waters
					1200	Siwa coastal waters & estuary of Cenranae River
					>1517	Kuricaddi River estuary, Maros & Bulungan coastal waters
OPA-02	18	4	14	4	450	Siwa coastal waters & Cenranae River estuary
					660	
					900	
					>1517	Kuricaddi River estuary, Maros & Bulungan coastal waters

Specific or unique alleles in each zone indicate differential evolution may be occurring in the Makassar Strait and Gulf of Bone populations as an adaptation to environmental conditions. *Lates calcarifer* is a species which does not generally migrate far either within or between river systems, a fact which is thought likely to explain the development of specific alleles within each population or region. In addition, fishing pressure, land-use within the watershed, predators, nutrition and food availability, and other environmental factors are variables which can cause mutations which could

influence the genetic variation in Asian seabass at each site. Frost et al. [15] add that the effective population size, the relative contribution of different potential parents, site-based differences in larval and juvenile survival rates during metamorphosis, can all affect genetic variation.

Figure 2 shows the spread of genetic distance between individual Asian seabass in each population. In general, the within-population genetic distance spread is greater in Asian seabass from the Gulf of Bone than in those from the Makassar Strait. The greater genetic distance between individuals in the Gulf of Bone indicates that there may be a speciation phenomenon occurring in the Asian seabass population in the waters of the Gulf of Bone. The speciation model that could explain this phenomenon is sympatric speciation, which refers to the process in which two or more species of descendants originate from one ancestral species in the same geographical location [16].

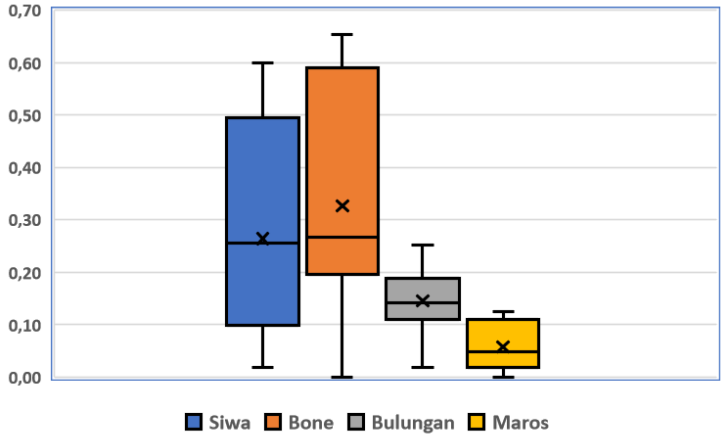

Figure 2. Intra population genetic distance spread of Asian seabass (*Lates calcarifer* Bloch, 1790)

Table 3 presents data on the genetic distance between four Asian seabass populations in the Wallacea region. The greatest genetic distance (0.291) was between Asian seabass populations in the coastal waters of Bulungan in North Kalimantan (Makassar Strait) and the Cenranae river estuary in Bone Regency (Gulf of Bone), while the lowest (0.086) was between the Bulungan and Maros populations, on opposite sides of the Makassar Strait. This information should be very useful in designing an Asian seabass breeding program to produce seeds with high fitness and adaptability. Macbeth et al. [17] suggested that much could be gained if a new breeding program was initiated by assessing the genetic variation of the wild stock because different strains would be more suitable for commercial scale production. The walk-back selection program proposed by Robinson et al. [18] has not yet evaluated the potential benefits of using multiple broodstock strains from different geographic locations. Breeding programs taking different strains from different regions and locations have been implemented for several farmed fish species. For example, breeding programs using six *Labeo rohita* strains showed a difference of 52% slower and faster growing fish [19]; a breeding program using five *Oncorhynchus mykiss* strains gave a 73% difference in weight parameters [20]; and a breeding program between Abbassa and Maryout tilapia produced differences of 104% in body weight [21].

Table 3. Paired genetic distance between four Asian seabass populations

Population	Siwa	Bone	Bulungan	Maros
Siwa	0.000			
Bone	0.237	0.000		
Bulungan	0.232	**0.291**	0.000	
Maros	0.223	0.268	**0.086**	0.000

The phylogenetic analysis shows that Asian seabass from the coastal waters of Maros in South Sulawesi and Bulungan in North Kalimantan form a single Makassar Strait clade. This is well separated from the Gulf of Bone clade comprising Asian seabass from the waters of Siwa Beach, Wajo Regency and the Cenranae River estuary in Bone Regency (Figure 3).

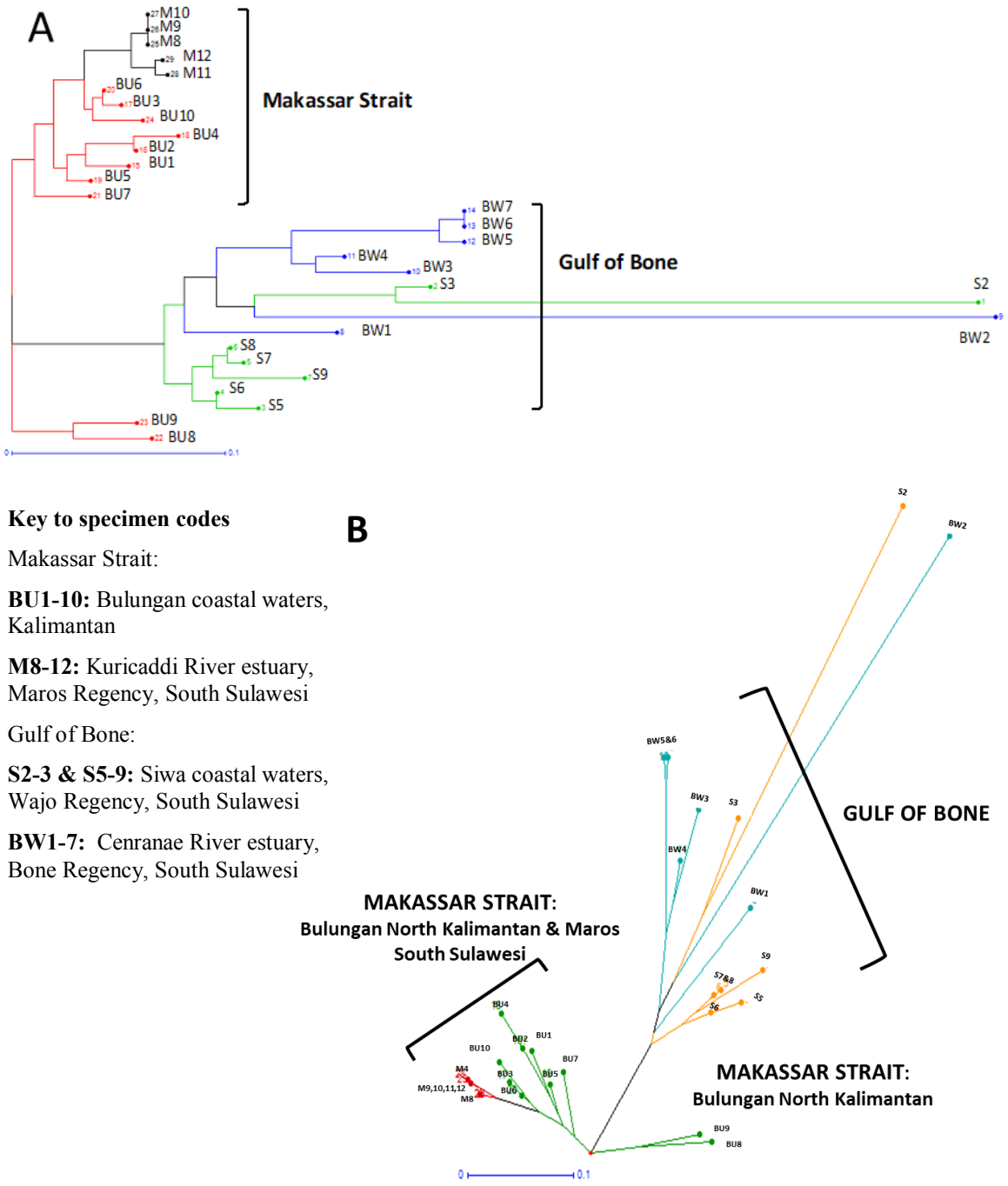

Figure 3. Phylogenetic relationships between 29 Asian seabass *Lates calcarifer* specimens from the Makassar Strait and Gulf of Bone (Neighbor-joining with 500 bootstrap repeats). A (rooted tree) and B (unrooted tree) indicate the relatedness and evolutionary relationships between individuals.

The geographical distribution and differential levels of gene flow within and between seaways are considered the most likely factors explaining the formation of these two clades. Slatkin [22] explained

that the level of gene flow is determined, among other things, by gamete and zygote dispersal and reproductive success. The larvae of Asian seabass are pelagic [23]. Planktonic larvae of many marine species can survive for months at sea and are dispersed passively by ocean currents. Although tracking a single larva is not possible, the capacity for long distance distribution suggests that long-distance gene flow from one population to another is common [22]. Although several studies have shown that juvenile and adult *L. calcarifer* do not migrate extensively [24], gene flow between the Asian seabass populations in North Kalimantan and Maros, South Sulawesi is likely to occur through the spread of gametes, zygotes, and larvae. One interesting results of this study is the indication that the Asian seabass population in Bulungan, North Kalimantan, may have come from two different ancestral groups, as there are two individuals from this population which form a separate clade. The scale of the gene flow determines the amount of evolutionary distance [22].

Geographical barriers are thought to be the cause of the separate clades formed by Asian population seabass from the Gulf of Bone and the Makassar Strait. The results indicate genetic differentiation in *L. calcarifer* resulting from isolation by the landmass of South Sulawesi as well as distance. Geographical barriers can cause reproductive isolation so that a lack of gene flow is suspected between Asian seabass in the Makassar Strait and those in the Gulf of Bone. Geographical barriers could also lead to an accumulation of morphological differences, as reported by Irmawati et al. for Asian seabass in the Gulf of Bone and Makassar Strait [8].

4. Conclusions

Based on the analysis of genomic DNA variation in the Asian seabass *Lates calcarifer* using the PCR-RAPD method and discussion, it can be concluded that this method was able to detect a population structure that is significant in terms of both evolutionary patterns and demographics. Genetic distance and the presence of unique/specific alleles provide important information for designing a breeding strategy. Cross breeding between Asian seabass from the Makassar Strait and the Gulf of Bone can be recommended to produce commercial-scale seeds with broad adaptability. However, further research to analyse and characterise unique alleles and genetic diversity with respect to productivity-related characteristics would further complement the Asian seabass breeding program data base.

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