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論文内容要旨

論文題目

Influence of hatchery protocols on skeletal malformation and genetic characteristics of Asian seabass *Lates calcarifer*

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Introduction

Lates calcarifer is one of the species of the family Latidae, commonly known as barramundi in Australia or Asian seabass in South-East Asian countries. This species can be found in coastal and brackish water areas of the tropical Indo-West Pacific region, including the Persian Gulf to India, Taiwan stretching to Papua New Guinea and Northern Australia. *L. calcarifer* is a protandrous hermaphrodite species that matures as a male first and turns into a female when it reaches a specific body size. As a euryhaline species, *L. calcarifer* spawns in seawater and grows mainly in brackish water areas. *L. calcarifer* inhabits coastal marine and estuarine to freshwater habitats, adults are mainly in estuaries, and younger fish are commonly found in freshwater. *L. calcarifer* is one of popular coastal aquaculture species in Indo-Pacific region including Cambodia. In Cambodia this species has been identified as main target species, since about 64% of coastal aquaculture farmers are raising this species as primary source. Despite the high demand for seabass offspring (3 million/year), Cambodia can produce only 10% of total requirement domestically. However, the observed gap between demand and domestic production, suggests a potential for increased production to meet the market needs for *L. calcarifer* offspring. Through some previous reports, unsuitable physicochemical environment, malnutrition, disease, poor rearing technique, and low genetic diversity were identified as possible causes in producing the undesirable characteristics in hatchery-raised-offspring, which commonly have been practiced by many hatcheries, including *L. calcarifer*.

To improve the efficiency of offspring production, the influence of hatchery protocols on skeletal malformation and genetic characteristics of *L. calcarifer* was revealed from the broodstock and hatchery-raised-offspring in a Cambodian hatchery.

Chapter 1: Current status of osteological malformation in offspring of Asian seabass *Lates calcarifer*, raised in Cambodian hatchery

Osteological malformations have been reported in various aquaculture fish species, with occurrences commonly observed in different regions of the fish body such as jaws, opercula, vertebrae, and fins. In *L. calcarifer*, some deformity types have been documented, but the status and degree of these malformations have not been clearly categorized and described. Moreover, previous reports on malformations in hatchery-raised-offspring often lack adequate information on rearing conditions and hatchery operation protocols. The present study aims to address this gap by providing a comprehensive categorization of osteological malformations in *L. calcarifer* offspring raised in a Cambodian hatchery. This detailed categorization serves as a foundation for further studies on the subject.

The current status of osteological malformations in the offspring of *L. calcarifer* raised under the protocol of Cambodian hatchery operation was examined by using soft X-ray radiograph and double stained specimens.

As a result, the occurrence of malformations was observed in various regions of the fish body such as the lower jaw, vertebral centrums and spines. However, no significant difference was observed at the different fish ages ($p>0.05$). The cumulative rates of these malformations, compared to normal offspring, were 30.67±3.21%, 27.33±3.06%, and 31.00±4.36% on the 30th, 45th, and 60th days after hatching, respectively. Lower jaw malformations were classified into four types, including asymmetric, projected, knobby, and perforated. Various expressions for jaw malformations, such as pinched, twisted, elongated, and split, were documented in this species. Among these, only the asymmetric type (present study) resembled what was reported in previous studies. In vertebrae, three types of malformations, compression, fusion, and curvature were identified with rates ranging from 16.33±6.08~19.67±1.52%. Vertebral curvature is a rather severe malformation. Among three types of vertebral malformation, fusion and curvature overlapped with previous reports. Malformations in vertebral spines were predominantly observed as supernumerary, fusion, and compression at both neural and haemal spines, with rates ranging from 19.66±3.21% to 23.67±6.11%. Various types of vertebral and spinal deformities often co-occurred. Regarding to spinal malformation, several types, namely paired heamal arch neural spine fragment, broken neural spine, and paired haemal spine reported in hatchery-reared *L. calcarifer*. Of these, only supernumerary in haemal spine malformation type found in present study.

The present study provides the first detail description insight into the current status

of osteological malformations in the offspring of *L. calcarifer* under the protocol of Cambodian hatchery. This precise categorization serves as a foundation for further studies on the subject.

Chapter 2: Osteological malformation in offspring of Asian seabass *Lates calcarifer*, reared under different salinity condition

Salinity is as one of the most influential abiotic factors in the life of marine and brackish water organisms. However, the specific influence of salinity on skeletal malformation in some species is limited. In the case of *L. calcarifer*, existing literature has primarily focused on the morphology and occurrence of malformations in the jaw, opercula, and spine of cultured larvae. The intention of this study is to elucidate the effect of different salinity rearing conditions on skeletal malformation of *L. calcarifer* up to 60 days after hatching (DAH). The findings are crucial for enhancing the operational efficiency of hatcheries producing offspring and potentially expanding nursery operations in freshwater and brackish water.

The osteological malformation types of in offspring of *L. calcarifer* reared under different salinity condition (30 PSU, 15 PSU, and 0 PSU) at 30th DAH, 45th DAH and 60th DAH were examined. The acclimation of fish to desirable salinity condition was carried out at a rate ranging from 0.5 to 2 PSU/day. Treatment 1, salinity about 30 PSU was maintained from the beginning until the end of experiment. Treatment 2, the salinity was gradually decreased to 15 PSU at 15-day-old fish, while Treatment 3, the salinity was decreased to 0 PSU at 30-day-old fish. The skeletal malformations of specimens were evaluated by using soft X-ray radiograph and double stained specimens.

As a result, all skeletal malformation types, including lower jaw, vertebrae, and vertebral spines, were observed across different salinities and fish ages. Total malformation rates showed a significant difference at various salinity conditions ($p<0.05$), but no significant difference was observed among ages ($p>0.05$). At 15 PSU, the higher significance was observed at 45th DAH (45.66±4.04%) and 60th DAH (51.66±4.73%). Lower jaw malformation, Asymmetric, Projected, Knobby, and Perforated rates did not show significant difference under various salinity conditions, but it exhibited significant across different ages, with the lower rates (6.33%) in 15 PSU group at 45th DAH. In some species, various constant salinity conditions were reported as an inducing factor on jaw malformation during incubation to completion of yolk resorption stage, including *Anguilla japonica*, *Ophiodon elongatus*, and *Hippoglossus hippoglossus*. Regarding to experiment protocols, suggest that jaw malformation in previous studies may affect during the autotrophic rather than exotrophic. Vertebral malformation, compression, fusion, and curvature showed a significant difference under various salinity conditions

and fish ages. At 15 PSU, lower rates of vertebral malformation were observed at 30th DAH (18.33±4.03%), while higher rates were noted at 45th DAH (38.33±7.02%) and 60th DAH (38.67±1.15%). At 0 PSU, lower rates were observed at 60th DAH (9.67±1.15%), whereas higher rates were reported at 30th DAH (25.00±2.65%) and 45th DAH (20.00±5.20%). However, no significant difference was observed at 30 PSU. Vertebral spine malformations, including compression, supernumerary was significantly influenced by different salinity conditions, but it did not show significant effect at different ages. Higher rates were observed in 15 PSU at 45th DAH (40.67±5.86%) and 60th DAH (43.00±3.46%), while lower rates were observed in 30 PSU and 0 PSU. The finding in the present study, suggests that acclimation *L. calcarifer* larvae in early stage from higher to lower salinity affected on vertebral and spinal malformation. Previous studies stated that, the influence level of salinity on vertebral curvature is various by species. In *O. elongates* larvae, the higher rate of malformation was observed at 35 PSU, in *H. hippogossu* larvae was at 34 PSU, and in *A. japonica* larvae was 42 PSU. In some species, higher vertebral curvature rates were reported in both lower and higher salinities, such as *Parophrys vetulus* and *Polydactylus sexfilis*. For other malformation types, the higher rates were reported in both lower and higher salinity in *O. elongates* larvae.

The vertebral and spinal malformation occurred across various salinity and ages, suggests that not only salinity but also other factors may be involved as causes of vertebral and spinal malformation in present study; thus, further research is required to verify those factors.

Chapter 3: Genetic characteristics of cultured broodstock and their offspring of Asian seabass *Lates calcarifer* inferred from mtDNA analysis

Possible causes of low genetic diversity in broodstock were reported, commonly has been practiced in many *L. calcarifer* hatcheries by using repeatedly subcultured broodstock and small number of fish as broodstock in offspring production. Such practice may lead to severe population bottlenecks. Since wild *L. calcarifer* are rare in Cambodia, the source of broodstock is limited. The Cambodian hatchery obtained adult fish from private farms in Koh Kong and Preah Sihanouk, most likely subcultured fish originating from Thailand. Likewise, the present study aims to characterize the genetic features of *L. calcarifer* broodstock in the Cambodian hatchery through mtDNA analysis for selecting suitable strain.

Genetic characteristics of *L. calcarifer* broodstock, 49 specimens from two farms (Koh Kong and Preah Sihanouk provinces) and 27 from a Cambodian hatchery, and their offspring, 41 from three batches were examined by using mitochondrial DNA analysis. TNES-Urea buffer method (Asahida et al, 1996) was used to isolate total cellular DNA. A part of COI region was PCR-amplified from total cellular DNA using reported primers (Vij et al., 2014). PCR products were sequenced using the BigDye. The nucleotide

sequences were analyzed by MEGA 7.0.26 (Kumar et al., 2016) software. Genetic diversity between the population of broodstock and their offspring was examined by using Arlequin software (ver. 3.5). Obtained sequences were compared with reported sequences of *L. calcarifer* in GenBank.

Four haplotypes (Hap) were identified through 535 bp of the COI region. Hap 1 was detected in all the batches of offspring and most of the broodstock individuals, followed by Hap 2, Hap 3, and Hap 4. Moreover, phylogenetic trees, maximum likelihood and neighbor-joining were constructed and categorized into Group 1 (Hap 1, Hap 3, and Hap 4) and Group 2 (Hap 2). To determine the genetic distance, sequences in the present study were compared with the sequences of *L. calcarifer* from GenBank. As a result, Group 1 and sequences from Indonesia, Malaysia, Singapore, Thailand, and Australia showed a high divergence from Group 2 and sequences from India, Bangladesh, and Myanmar. Furthermore, Pethiyagoda and Gill (2012) described two new species of *Lates*, *Lates. lakdiva* and *Lates. uwisara* based on morphological analyses. However, discrepancies between morphological and genetic data raised questions about the accuracy of the original species descriptions and the possibility of hybridization between *L. calcarifer* and *L. uwisara* is considered. Individuals in Group 2 may belong to another species. Thus, the hatchery needs to distinguish species to maintain good strains for offspring production. Within Group 1, no genetic diversity was observed in all the batches of offspring and hatchery-raised broodstock, while the farm-raised broodstock showed very low genetic diversity. The result from this study will be useful for selecting suitable strain for offspring production.

The present study highlights the critical role of the operational protocol in both the quality and quantity of *L. calcarifer* offspring. It reveals a spectrum of malformations, ranging from mild to severe, in the lower jaw, vertebrae, and spinal regions within the context of the Cambodian hatchery operation protocol. The study also reveals the impact of various salinity conditions on skeletal malformations. However, many potential causes of skeletal malformations remain, underscoring the need for further exploration in future studies. Furthermore, the genetic characteristics of the broodstock used in Cambodian hatcheries were inferred. In light of these findings, several measures must be undertaken to enhance the efficiency of offspring production in Cambodian hatcheries.

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