Reproductive and Genetic Studies in Large Yellow Croaker

Reproduksjon og genetikk hos den marine fiskearten *Larimichthys crocea*

Philosophiae Doctor (PhD) Thesis

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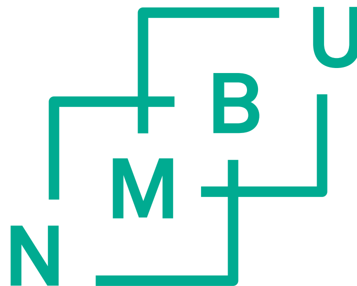

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SUMMARY

Large yellow croaker (*Larimichthys crocea*) is distributed in the coastal regions of East Asia and is one of the most important marine aquaculture species in China. However, due to overfishing, it has been difficult to capture wild fish for the last decades. Artificial reproduction and aquaculture of it has been performed since 1985 in China, but important production traits have been deteriorating due to poor broodstock practices. It is thus considered necessary to initiate a modern breeding programme to improve these traits through an efficient selection programme, and the facilitations of this thus became the aim of this PhD project.

In paper I, two trials with artificial fertilisation in large yellow croaker were tested and compared. Results show that double injections of LHRH A_3 (dosage 0.8 μg/kg and 2 μg/kg) in females with an interval of 10 h, and single injection (dosage 1μg/kg) in males gave highest fertilisation and hatching percentage. The best latency time (a time period between first hormone injection and spawning) was 29.5-35 h, which were determined by only monitoring courtship behaviour of males to reduce stress. Compared to the initial trial, the percentage of females with spawning difficulties decreased from 30% to 10%, while the fertilisation rate and hatching rate increased from 28% to 41% and from 52% to 62%, respectively. The new protocol of artificial fertilisation will be useful in family construction of future breeding programmes.

In paper II, phenotypic and genetic parameters were estimated for body weight, body length and body height in six months old large yellow croaker. The trial was planned to run until the fish had reached a normal harvest size, but due to severe damages to the test facility during a typhon, the trial had to be ended prematurely. The records used for parameter estimation thus are from when the fish were tagged, at an age of only 6 months. The estimates of heritability, which ranged from 0.31±0.06 to 0.41±0.07, may thus be influenced also by common environmental effects unique to each family prior to tagging. The three traits recorded were closely correlated, both genetically and phenotypically, with all genetic correlations above 0.74 and phenotypic correlations above 0.84. These results thus clearly indicate good prospects for efficient selection for BW, BL and BH in large yellow croaker.

In paper III, a high-density genetic linkage map was constructed using a family from a cross of the Mindong and Daiqu strains. A total of 20,147 single nucleotide polymorphisms (SNPs) markers were assigned to 24 linkage groups (LGs). The length of the consensus linkage map was 1757.4 cM, with individual LGs ranging from 51.9cM (LG6) to 124.6cM (LG9). Sex-specific maps were also constructed, and recombination events occurred 20% more frequently in females. Collapsed or co-occurring markers in the genetic maps were re-ordered according to their relative positions in the ASM435267v1 genome assembly to produce integrated linkage maps, resulting in 9,885 SNPs distributed across the 24 LGs. The recombination patterns of most LGs were sigmoidal, with higher recombination rates in the middle and suppressed recombination rates at the ends, consistent with sub-telocentric and acrocentric chromosomes, respectively. The average recombination rate in the integrated female and male maps was respectively 3.55 cM/Mb and 3.05 cM/Mb, as recombination rates in the females were higher than in the males for most LGs. No significant quantitative trait loci (QTL) for growth traits at six months were detected in the QTL or association analyses. The study indicates there may be genetic differences between the Daiqu strain and Mindong strain that can be important for application of genetic tools in a mixed breeding population.

SAMMENDRAG

Fisken med det engelske navnet large yellow croaker (*Larimichthys crocea*) er utbredt i de kystnære områdene i Øst-Asia og er en av de viktigste marine oppdrettsartene i Kina, men i den senere tid har det vært svært lite fangst av villfisk. Kunstig reproduksjon og oppdrett av denne arten har i Kina vært praktisert siden 1985, men på grunn av dårlige avlsrutiner har produksjons¬egenskapene etter hvert blitt dårligere. Det er derfor nødvendig å initiere et moderne avlsprogram for å forbedre disse egenskaper gjennom systematisk seleksjon, og dette doktorgradsprosjektet har hatt som mål å forberede et effektivt avlsprogram for tilvekstegenskaper for large yellow croaker.

I artikkel I er det beskrevet forbedrede prosedyrer for kunstig reproduksjon og hold av separate familier, noe som er nødvendig for å kunne starte et systematisk avlsprogram. En metode for stegvis forbedring av prosedyrene har blitt brukt, og det beste resultatet ble oppnådd medå injisere hunnene to ganger med kjønnshormonet LHRH A3, med et intervall på 10 timer, og en dosering på henholdsvis 0.8 µg/kg og 2 µg/kg. Hannene ble hormonbehandlet kun en gang. Tiden mellom behandling og frem til optimal eggmodning ble funnet å være 30 – 35 timer. Riktig tidspunkt ble bestemt ved å observere hannens oppførsel, som med en karakteristisk lyd indikerer at hunnen er klar. Andelen av hunfisk med dårlig eggkvalitet var kun 10 %, mens befruktningsprosenten var 41 % og klekkeprosenten av befrukta egg var 62 %. Metodene som her er foreslått vil derfor være egnede som et utgangspunkt for videre forbedring av rutiner for modning og behandling av stamfisk, og de gjør det mulig å starte et avlsprogram for denne nye oppdrettsarten.

I artikkel II ble vekstrelaterte egenskaper, dvs. vekt, lengde og høyde, undersøkt hos seks -måneder gammel large yellow croaker og fenotypiske og genetiske parametere ble beregnet. Arvegraden for disse egenskapene varierte fra 0.31 ± 0.06 til 0.41 ± 0.07. De tre egenskapene var klart korrelert, både genetisk og fenotypisk. De genetiske korrelasjonene var alle over 0,74, mens de fenotypiske alle var over 0,84. Disse resultatene viser at det er gode muligheter for å selektere for egenskapen tilvekst i denne arten.

I artikkel III presenteres et mer nøyaktig koblingskart for large yellow croaker. Det presenteres både et felles koblingskart for begge kjønn og et hann- og hunn-koblingskart.

Kartene er konstruert ved hjelp av såkalt «Restriction-site associated DNA» (RAD) -sekvensering. Det ble også sett etter markører som kan være knyttet til gen som styrer vekstegenskapene. Lengden på koblingskartet for begge kjønn var 1757 cM. Det ble også gjort en genetisk analyse for å søke etter markører som er koblet med disse egenskapene, såkalte QTLs, i 6 måneder gammel fisk. Videre ble kjønnsspesifikke kart konstruert, og vi fant at rekombinasjonshendelser forekom 20 % hyppigere hos hunfisken. Gjennomsnittlig rekombinasjonshyppighett var henholdsvis 3,55 cM/Mb for hunfisk og 3,05 cM/Mb for hanfisk. Ingen signifikante markører for kvantitative egenskaper (QTL) for de undersøkte egenskapene ble påvist i i denne studien. , men det ble funnet klare indikasjoner på at det kan være betydelige genetiske forskjeller mellom Daiqu-stammen og Mindong-stammen.

ABBREVIATIONS

AFLP - Amplified Fragment Length Polymorphism

BH - Body Height

BL - Body Length

BLUP - Best Linear Unbiased Prediction

BW - Body Weight

EBV - Estimated Breeding Values

FOM - Final Oocyte Maturation

FSH - Follicle-Stimulating Hormone

GBLUP - Genomic Best Linear Unbiased Prediction

GBS - Genotyping By Sequencing

GnRHa - Gonadotropin-Releasing Hormone agonist

GS - Genomic Selection

GWAS - Genome-Wide Association Study

HDPE - High-Density PolyEthylene

IUCN - The International Union for Conservation of Nature

LD - Linkage Disequilibrium

LG - Linkage Group

LH - Luteinizing Hormone

LHRH A_3 - Luteinizing Hormone Releasing Hormone A_3

LOD - Logarithm of Odds

MAF – Minor Allele Frequency

MAS - Marker Assisted Selection

NCBI - US-based National Center for Biotechnology Information

OCS - Optimal Contribution Selection

PIT - Passive Integrated Transponders

QTL - Quantitative Trait Loci

RAD Seq - Restriction-site Associated DNA Sequencing

SNP - Single Nucleotide Polymorphism

SSR- Simple Sequence Repeat

LIST OF PAPERS

The thesis is based on the following papers. The reference to these papers is given by their Roman numerals.

Paper I:

Xinxiu Yu, Changwen Wu and Hans Magnus Gjøen. (2017).

Artificial Fertilisation of Large Yellow Croaker (*Larimichthys crocea*) and Generation of Families for a Selective Breeding Programme. International Aquatic Research, 4. doi:10.1007/s40071-017-0164-3

Paper II:

Xinxiu Yu, Tormod Ådnøy, Zhenming Lv, Changwen Wu and Hans Magnus Gjøen. (2020).

Phenotypic and Genetic Parameter Estimation for Growth Traits in Juvenile Large Yellow Croaker (*Larimichthys crocea*). Fisheries and Aquaculture Journal. 11:274. doi: 10.35248/2150-3508.20.11.274

Paper III:

Xinxiu Yu, Rajesh Joshi, Hans Magnus Gjøen, Zhenming Lv and Matthew Kent.

Construction of Genetic Linkage Maps from a Hybrid Family of Large Yellow Croaker (*Larimichthys crocea*). *Submitted to Frontiers in Genetics, under interactive review.*

1. GENERAL INTRODUCTION

1.1 Aquaculture of large yellow croaker

Large yellow croaker (*Larimichthys crocea*) is a carnivorous marine fish species belonging to the *Sciaenidae* family (**Figure 1**). The fish typically live in the mid-water zone at a depth of 10-60 m in the southeast coastline of China. The optimum growth temperature for this species is 18-25 °C. When the water temperature is below 13°C or above 30°C, the feed intake is significantly reduced. Additionally, the adaptable salinity is 25-35‰, the suitable pH is 7.9-8.4, and the dissolved oxygen should generally be above 4 mg/L (Liu, 2013). The fish is a multiple spawning fish with asynchronous ovarian development (Gong et al., 1986). Both the male and female fish can emit croaking or drumming sounds by contracting sonic muscles and vibrating bladder during courtship and spawning season (Lo, 2011). The fish is a good resource of excellent nutritional value, providing high quality protein and the healthy omega-3 fatty acids.

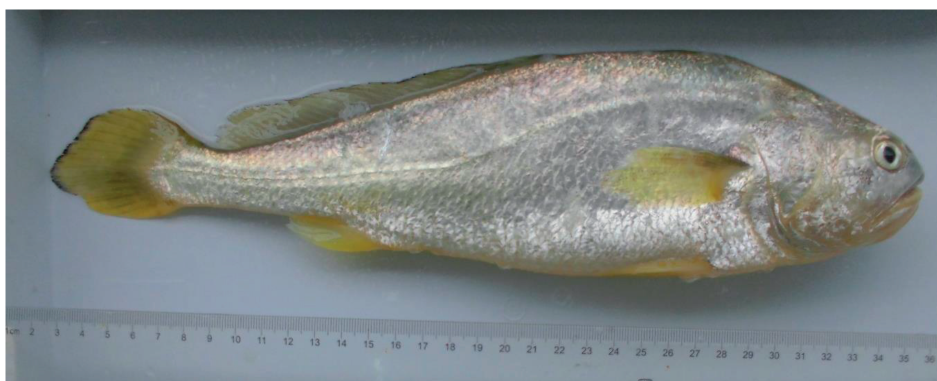

Figure 1. Large yellow croaker (*Larimichthys crocea*).

Three geographic strains were initially identified in the coastal waters of China: Daiqu strain, Mindong strain and Naozhou strain (**Figure 2**), based on morphological and biological characteristics, such as numbers of gillrakers and spawning season (Tian et al. 1962; Xu et al. 1962). Daiqu and Mindong are the two major farmed strains, and there are some differences between them based on the current research: the contents of total amino acid and essential amino acid in dorsal muscles of Daiqu strain have been found to be significantly higher than that in the Mindong strain (Li et al., 2009); the genetic diversity of Daiqu strain was in two studies higher than in the Mindong strain (Liu et al., 2015; Huang et al., 2011); and the Daiqu strain had better performance in body shape (Huang et al., 2006), and had higher resistance to low temperatures with 22.5% higher survival rate at 6°C than Mindong strain(Miao et al., 2014).

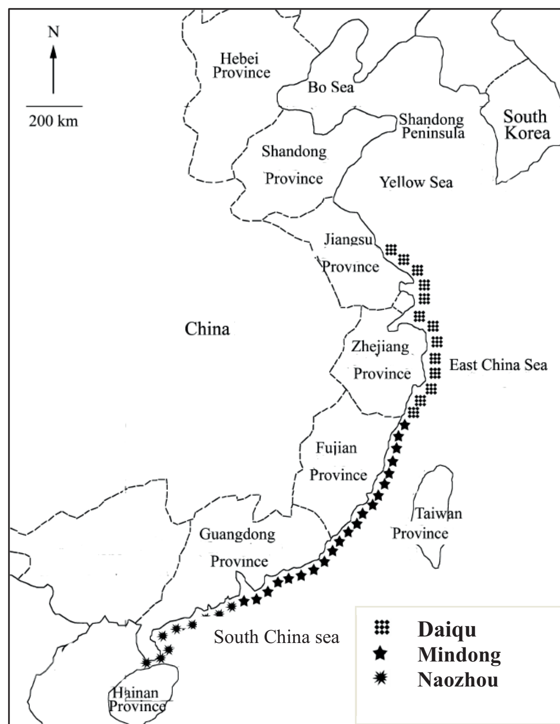

Figure 2. Geographic distribution of the large yellow croaker in coastal waters of China. (Modified based on Liu and Mitcheson (2008))

The fish used to be one of the four major marine fisheries species in China. The annual catch of large yellow croaker in Fujian province from 1956 to 2015 is shown in **Figure 3** (Ye et al., 2020). A decrease fishery of this species also occurred in other coastal regions (Zhang et al., 2017). The wild fishery resource has thus been nearly extinct since the 1980s, due to overfishing and environmental deterioration (Ye et al., 2020; Liu and Mitcheson, 2008). As there is no evidence that this species is being sustainably exploited, it is listed as Critically Endangered by the International Union for Conservation of Nature (IUCN) Red List of Threatened Species (Liu et al., 2020). Thus, it is urgent to develop effective artificial cultivation to both supply juvenile fish for producers and create an opportunity for restocking the wild population.

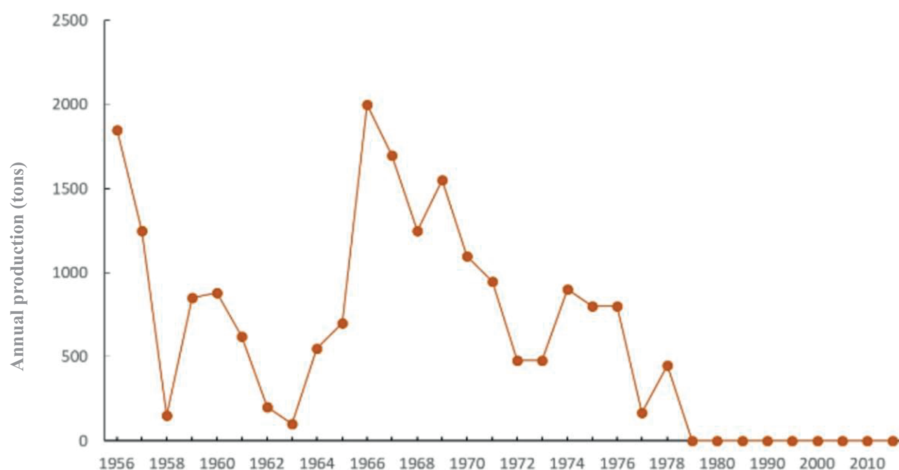

Figure 3. Annual production of the wild-caught large yellow croakers from 1956 to 2015 in Guanjingyang, Fujian province, China. (Ye et al., 2020)

The artificial breeding of the Mindong strain started in 1985, whereas the breeding of the Daiqu strain started in 1999 (Chen et al., 2018). The farming is concentrated in the southeast sea area of China, including the Fujian Province (mainly Mindong strain), Guangdong Province (mainly Mindong strain) and Zhejiang Province (mainly Daiqu strain), where the fish are cultured in cement tanks from fries to juveniles, and after one month in the nursery, the juvenile fish (ca. 3 cm body length) are transferred to sea cages. The juvenile fish production and grow-out production have been increasing since 2012 (**Figure 4**). The Fujian Province is the core breeding and farming area, covering more than 85% of the fry production. In 2017, the total harvest was 177,600 tons, of which

150,542 tons (85%) were produced in Fujian Province, 12,500 tons (7%) in Guangdong province and 14,600 tons (8%) in Zhejiang Province, according to FAO Statistics (https://www.fao.org/in-action/globefish/market-reports/resource-detail/en/c/1189979/). The production continued to increase in 2019, over 220,000 tons, accounting for more than 12% of the cultured marine fish production in China. Now large yellow croaker thus again has become a commercially important fish, having the largest production of the marine aquaculture species in China. The annual production value of large yellow croaker culture exceeds 10 billion RMB (about US$ 7 billion), and the value of the exported volume is over US$ 100 million (Chen et al. 2018). According to the World Bank (2013), China will consume 38% of the global fish production in 2030, and the aquaculture of large yellow croaker therefore will be an important contributor to meet the future market demand. Thus, the industry has a considerable potential to be lucrative for those aquaculture farmers that are able to deliver.

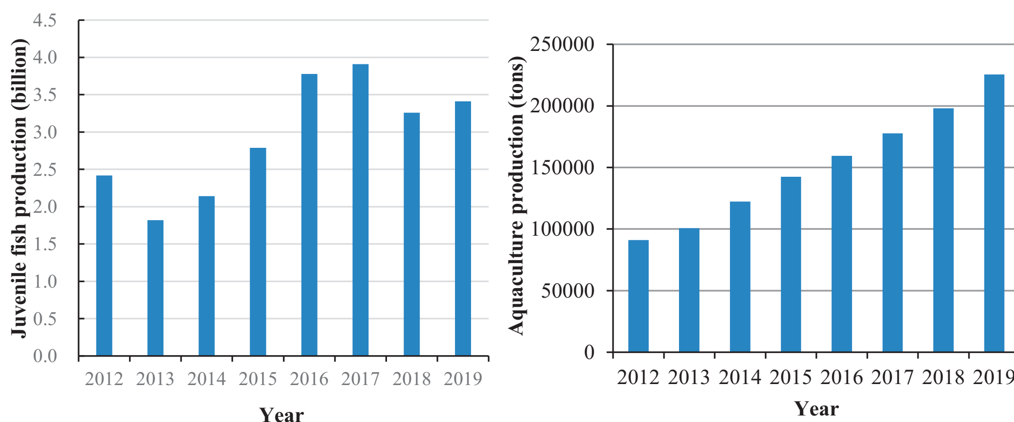

Figure 4. Juvenile fish production (left) and total production of large yellow croaker (right) per year. (based on China Fishery Statistical Yearbook, 2013-2020)

Unfortunately, the production of cage-based aquaculture in southeast China is frequently threatened by typhoons, which can cause severe economic losses. Over 95% of the aquaculture production is based on traditional cages (normally 3m×3m to 5m×5m nets with 4-5m depth), which are made of materials that are easily damaged in a typhoon (**Figure 5**). For example, in 2018, the super-typhoon *Maria* destroyed over 10% of the cages in Fujian province, with approximately 23 million US dollars of economic loss in aquaculture production (https://kknews.cc/zh-sg/society/mo95592.html). Another challenge is that these cages have to be installed in the inshore water where the seawater exchange rate is relatively low. Thus, residuals of feed are accumulated at the seabed, and fish disease outbreaks are common due to poor water quality. Offshore cages or deep-sea cages (diameter 20m, depth 15m) are an attractive alternative to resist typhoon storms. Compared to traditional sea-cages in bays, the offshore or deep-sea cages in semi-open sea areas have obvious advantages: not only expanding culture areas and resisting the destruction of typhoon storms but also providing a better aquaculture environment and enhancing the quality of the fish

Figure 5. Traditional sea cages (**a**) Before typhoon; (**b**) After typhoon
(**b** source: http://www.muwangjt.com/web_UploadFile/image/20190812/20190812100214461446.jpg)

products. The quality of fish cultured in deep-sea cages is reported to be similar to its wild counterpart (Guo et al. 2018). HDPE cages are one of the offshore cages introduced

from Norway (**Figure 6)**, which have become more commonly used in the aquaculture of large yellow croaker. These offshore cages can increase the output of high-quality fish and income for fishermen, becoming the main force for the sustainable development of mariculture (Chen et al. 2018).

Figure 6. HDPE cages (source: http://www.tradekey.com/product-free/Hdpe-Cage-For-Fish-Farms-736231.html)

1.2 Deterioration of economic traits in farmed large yellow croaker

Generally, the mismanagement of genetic resources and diversity during the domestication of many aquaculture species has led to reduced genetic resilience and the subsequent occurrence of diseases caused by crowding of farmed species (Houston et al., 2020). Similarly, with the rapid expansion of the mariculture, large yellow croaker has in recent years suffered from serious deterioration of economically important traits, such as slow growth rate, low resistance to diseases and too early sexual maturity, all hindering sustainable development of this important aquaculture industry. Due to the high fecundity of large yellow croaker, a relatively small number of broodfish are used in each generation, as the selection is only done based on own phenotype without pedigree information. As a result, inbreeding depression is accumulated rapidly, and genetic diversity is thus lost in the farmed stocks (Wu et al., 2011; Wang et al., 2012a).

Currently, approximately two years is needed to reach a market-sized fish of ca. 350 g (Chen et al., 2018). The slow growth rate causes high feed cost, which accounts for 74% - 84% of total costs in the culture of the fish (Liao et al. 2012). The slow growth

rate also creates a higher risk for the cultured fish to escape or die during the long production time, especially in traditional cages, as typhoons occur every year in southeast China. Early sexual maturation is also negative and detrimental to the growth rate, health, and quality of the fish. It was reported that females needed 3 years while males needed 2 years to get gonadal maturation in the wild fish (Zheng and Xu, 1977), however, Fang et al. (2000) studied the gonadal development in farmed fish and predicted that 50-60% females and 60-70% males had gonadal maturation already at 15 months of age, based on observations of milt flowing out by gentle pressure on the abdomen of 100-150 g male fish and enlarged ovaries of aprox. 200 g female fish .

During the long growth phase from fry to marketable size, large yellow croaker can suffer from over 20 common diseases, of which the most serious one is parasitic infestation (Wang et al. 2012b). The most devastating parasite, *Cryptocaryon irritans,* is a ciliate protozoan that parasitises large yellow croaker easily and causes white spot disease, particularly when water temperature stays high, i.e. between 20 and 25 °C. The mortality can then reach 100% (Sun et al. 2011).

Thus, with the increase in consumption and demand for high quality large yellow croaker, the interest in breeding programmes for this species is growing. Selective breeding is increasingly recognised as a key component of the sustainable production of aquaculture species (Robledo et al., 2017). Until now, no effective selective breeding programme has been applied in large yellow croaker, and it is therefore necessary to establish and innovate a modern breeding programme to increase fish productivity and quality, and to utilise available feed and water resources more efficiently.

1.3 Pedigree-based selective breeding in aquaculture

Directed selection offers the potential to improve fish productivity and quality, and is an important tool that has obtained an increased interest in the aquaculture industry lately. Selective breeding schemes have for a long time been widely recognised as an efficient means to increase livestock production, and remarkable genetic improvements and productivity increases have been achieved by efficient selective breeding programmes in farm animals (Schultz et al., 2020). Researchers in Norway pioneered the transfer of genetics knowledge from livestock to fish species. The main Norwegian selective breeding programme for Atlantic salmon (*Salmo salar*) has documented that an average genetic change per generation for growth rate is up to 13% at harvest size

(Gjedrem and Rye, 2018), and that the selected salmon grow twice as fast as wild Atlantic salmon in a period of only six generations of selection while requiring 25% less feed (Thodesen et al., 2006). Selective breeding programmes have also been carried out in other fish species, such as rainbow trout (*Oncorhynchus mykiss*) and Nile tilapia (*Oreochromis niloticus*) (Gjedrem and Robinson, 2014; Eknath et al., 1993; Bentsen et al., 2017). These results clearly show the great power and potential of selection to improve economically important traits in fish. Compared to farm animals, fish species have much higher fecundity, which enables breeders to produce large families, allowing for very high within family selection intensities. Family-based selective breeding programmes, using sibling information for invasive traits that cannot be recorded on the breeding candidate themselves, represent the industry breeding standard for fish species (Rye et al., 2010; Gjedrem et al., 2012).

For large yellow croaker, the breeding programme for economically important traits is still in its early stage, which limits further development of the large yellow croaker aquaculture industry. Currently, the selection of brood fish is based on phenotypes, mainly according to their growth performances, without incorporating any pedigree information. Phenotype selection can be effective on traits with high heritabilities, but due to high fecundity, it has the risk of quick accumulation of inbreeding, leading to loss of genetic variation and inbreeding depression, which is seriously causing a reduction in economically important traits, such as growth traits and disease resistance. To improve these traits genetically, the alternative way is to imply long term breeding programmes based on records from all relatives and pedigree information. With pedigree recording, there is considerable power to ensure high selection accuracy and fast genetic gains while keeping inbreeding at a low level.

1.3.1 Control of reproduction

To set up a pedigree-based selective breeding programme, a prerequisite is to master and control the reproduction, including artificial fertilisation and hatchery rearing, if conventional tagging systems are to be used (Refstie and Gjedrem, 2005). This usually also will require stripping the gametes of the fish, which can be difficult to do with many marine species. The main advantage of strip spawning and artificial fertilisation for breeding programmes is that it allows the design of different mating schemes in selection programmes. In particular, the use of a full or partly factorial design

enables the separation of additive, dominance, and maternal components of variance. Almost all commercial hatcheries producing large yellow croaker rely on spontaneous or natural spawning in groups, exceeding 100 fish, after hormone treatment, which complicates controlling and tracking genetic contributions from individual candidate breeders. Thus, the development of a protocol for artificial reproduction is crucial to implement family selection in large yellow croaker.

Large yellow croaker is a multiple spawner with asynchronous ovarian development. It often suffers reproductive dysfunction in the late stages of gametogenesis in a cultured environment, and usually, the females exhibit serious reproductive problems. Overall, this indicates that the captive environment in most instances is not optimal for successful spawning (Duncan et al., 2013), but the application of hormonal treatments has to a large extent effectively resolved the dysfunction problem in mass spawning. Frequently, luteinizing hormone-releasing hormone A_3 ($LHRHA_3$), an exogenous gonadotropin-releasing hormone agonist (GnRHa), is applied to synchronise ovulation and induce spawning in large yellow croaker. GnRH acts directly at the pituitary gland to stimulate follicle-stimulating hormone (FSH) and luteinizing hormone (LH) secretion that is released into the bloodstream to act on the gonads, where they stimulate the synthesis of gonad steroid hormones (**Figure 7**). GnRHa treatment induces final oocyte maturation (FOM) and ovulation in the ovaries and spermiation in the testis (Mañanós et al., 2009).

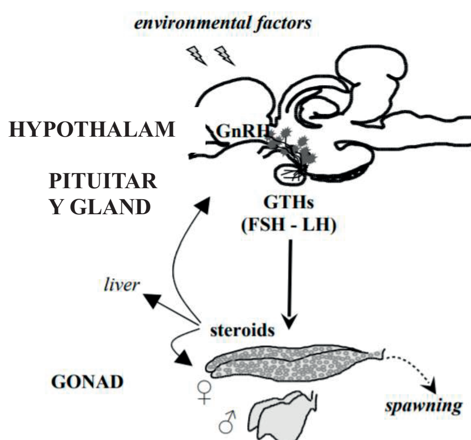

Figure 7. The brain-pituitary-gonad (BPG) axis, showing the critical hormones involved in the regulation of fish reproduction (Mañanós et al., 2009).

As noted above, large yellow croaker can spawn naturally in large groups (>100 fish) after hormone therapy in commercial production. In smaller groups (<10 fish), the oocytes will often be ovulated, but the eggs will in most cases not be released by the female (personal observation). Thus, egg stripping and artificial fertilisation are necessary to generate full-sib and half-sib families. Zheng et al. (2006) have described the basic procedures of artificial fertilisation in this fish species, but here still milt from different males was mixed together. Since sperm competition causes unequal contributions from males (Liu et al., 2012), these procedures are not satisfactory to construct enough families effectively for a family-based breeding programme. Furthermore, some important considerations in artificial fertilisation, such as the optimal stripping point after hormonal injections, were not given in Zheng's study. For artificial fertilisation, where eggs need to be hand-stripped, a correct latency period, which is a time period between first hormone injection and spawning, is essential to get the stripping time right. If stripping is done earlier than full ovulation, the fish will be stressed and the continued ovulation process may be stopped; if stripping is later than ovulation, the eggs will often be over-ripe and lose viability with time. The time after ovulation when eggs begin overripening was summarised in several fish species (**Table 1**). It seems that overripening proceeds much more rapidly in warm water species (Bromage et al., 1994). Large yellow croaker is also such a fish species, and it is vital to determine a correct latency time to obtain high-quality eggs for successful artificial fertilisation. It was previously reported that the latency time was about 48 h with a dose of 2 µg/kg LHRHA3 for females at a water temperature of 22-24°C (Zheng et al. 2006). This reported latency time was initially tested under similar conditions, but the over-ripening problem was found to be very severe, indicating that the knowledge of controlled artificial fertilisation was still insufficient. Thus, the stripping time and artificial fertilisation protocol should be revised and adjusted specifically to effectively generate families of large yellow croaker, which was the goal of paper I in this thesis.

Table 1. The post-ovulation time when eggs begin overripening in several fish species.

Species	Overripening	Spawning Temperature	References
White bass	30 min	22 °C	Mylonas et al., 1996
Striped bass	15-30 min	16-20 °C	Rottmann and Chapman, 1991
Common carp	50-80 min	18-24°C	Bromage et al., 1994
Nile tilapia	1 h	24 °C	Bromage et al., 1994
Atlantic halibut	4-6 h	4-7°C	Bromage et al., 1994
Rainbow trout	7 days	10 °C	Lahnsteiner, 2000
Atlantic salmon	6-8 days	5-8°C	Bromage et al., 1994

1.3.2 Genetic parameters

For each aquaculture species, preferably before the breeding programme is established, estimation of the genetic parameters should be carried out, in order to design more effective breeding programmes and give more accurate estimated breeding values (EBVs). Improving growth traits has been a major goal at the beginning of many breeding programmes in aquaculture (Janssen et al., 2017), as they have moderate-to-high heritability (h^2) and are easy to record on the large numbers of breeding candidates themselves (Fjalestad, 2005). For example, the heritability for the growth rate of salmonid fishes ranged from 0.2 to 0.3, and 10-15% genetic gain was obtained per generation in many programmes (Gjedrem, 2000). In the same way, growth improvement has been the highlighted trait in the initial breeding goal of large yellow croaker. However, up to date, the essential knowledge about quantitative genetic parameters for selective breeding is still limited in this fish species. Heritability estimates for growth traits have been reported in three studies, but all were based on relatively small sample sizes (Liu et al., 2011; Liu et al., 2013; Dong et al., 2016). For example, Liu et al. (2013) studied heritability for growth traits of 13 months old fish based on 959 offspring from 10 dams and 15 sires. Since knowledge about these important parameters of growth traits is still quite limited, there is an urgent need to run breeding experiments based on a larger population in order to obtain more reliable estimates of genetic parameters for economically important traits in this species, and

this was thus the goal of paper II in this thesis.

1.4 Use of genomic information in aquaculture selective breeding programmes

Selective breeding with pedigree information has been very successful in aquaculture species, and genetic gains are generally very high (Gjedrem and Rye, 2018). Furthermore, incorporation of genomic information into the selection process can improve selective breeding additionally in several ways, improving genetic gain and minimising inbreeding, especially for the invasive or “hard to measure” traits, such as disease resistance, feed conversion efficiency, environmental tolerance, and product quality. These traits are harder to select for because they are difficult to measure on the live breeding candidates, involve destructive sampling, or have low heritability (Zenger et al., 2019). Traditionally, the EBVs of these traits for breeding candidates are estimated based on phenotypes and EBV of their siblings, and not on their own phenotypes. Therefore, only the between-family genetic variances are utilised, whereas within-family genetic variations are missed out. Genomic tools such as those used to genotype SNPs, however, will allow breeders to access and utilise both between and within-family genetic variation, and thus, the accuracy of selection and genetic gain can be increased. Furthermore, traditional pedigree is strictly not needed, because the relationships between individuals can be calculated based on the genotypes by the genomic relationship which is more accurate. Thus, families do not have to be kept separate until tagging, which is a huge benefit, especially for species that spawn in groups and where physical family separation is challenging or impossible. It also removes the effect of common environment, *c,* unique to each family, and the proportion of variance caused by this effect, c^2 (equivalent to h^2), have in many cases been found to be up to 10% of the total variation. This effect is also often difficult to adjust for in the estimation of EBVs, since it is confounded with the genetic effect of families, and are thus often inflating the EBV estimates. This problem is especially pronounced when the breeding design does not allow for powerful separation of genetic and environmental effects, like the commonly used *nested* or *hierarchical* design (Berg and Henryon, 1998).

Although the uptake of genomic technology in aquaculture breeding has lagged behind terrestrially farmed animals (Robledo et al., 2017), the development and

application of sequencing and genotyping technologies has allowed aquaculture to narrow the gap. Genetic markers are necessary to distinguish between selection candidates by utilising the within-family genetic variation. Implementation of markers in the breeding programmes can be achieved in two main ways: marker-assisted selection (MAS) and genomic selection (GS). MAS and GS are complementary approaches to traditional pedigree-based breeding techniques.

1.4.1 Marker-assisted selection

MAS can be used to select favourable quantitative trait loci (QTL) alleles directly, but prior knowledge of the underlying QTL is required. QTL mapping and association mapping are two types of strategies to detect potential causal genes for quantitative traits. QTL mapping exploits the co-segregation of functional polymorphisms and adjacent markers within families or pedigrees of known ancestry. Association mapping or genome-wide association study (GWAS) detects direct effects of genetic variants or closely neighbouring genes in linkage disequilibrium in a randomly sampled population. QTL mapping and GWAS dissect phenotypes genetically, and then the associated molecular markers are used for MAS. In QTL mapping enabled by genetic linkage maps, the detected QTL regions can be quite large, and many genes can be identified as potential candidate ones. GWAS can increase mapping resolution, but may have a high rate of false positive arising from population structure and family relatedness (Kaler and Purcell, 2019). Therefore, the combination of QTL and GWAS analyses can compensate for the limitations of each approach (Korte and Farlow, 2013).

A striking example of MAS in aquaculture was the discovery of a QTL imparting resistance to Infectious Pancreatic Necrosis (IPN) in Atlantic salmon in Norway. The QTL accounted for about 80% of the total variation in this trait (Moen et al., 2009; Houston et al., 2012), and was used in selective breeding, leading to a dramatic reduction in IPN outbreaks (Norris, 2017). Another example is that the MAS programme succeeded in developing Japanese flounder resistance to lymphocystis disease (Fuji et al. 2007). MAS can be useful for some traits where major QTL have been identified, but most traits of economic importance in aquaculture species are polygenic and often have low heritabilities.

1.4.2 Genomic selection

Selective breeding methods in aquaculture have tended to evolve from the initial selection associated with domestication, to mass selection, family selection, marker-assisted selection, and now to genomic selection (Boudry et al., 2021).

Unlike QTL-based MAS, where the effect of each QTL is first tested for its statistical significance, the GS methods, first proposed by Meuwissen et al. (2001), do not require any prior knowledge of the underlying QTL, and estimate the effect of all genome-wide markers simultaneously through a prediction equation. GS consists of two main steps. First, a prediction equation is established in a training population, in which individuals are phenotyped and genotyped. Once a prediction equation is established, breeding candidates can then be selected based on their estimated genomic value, with or without phenotype records on those individuals. There are several approaches to implement GS, including Genomic Best Linear Unbiased Prediction (GBLUP) (Hayes et al., 2009), assuming all markers have the same weight, and Bayesian estimates (Daetwyler et al., 2010), allowing for variation of allelic effects of each marker, and assuming only a small number of them have a non-zero effect.

GS has revolutionised the selection in many farmed species, particularly in dairy cattle, pigs and major high-value crops (Boudry et al., 2021). Advances in genomic methodologies accompanied by reduced costs for analyses are enabling the increased use of GS in aquaculture. It has been used in commercially important aquaculture species, such as the Atlantic salmon (Tsai et al., 2016; Robledo, et al., 2018), and compared with pedigree-based prediction, the increased accuracy of genomic prediction has been evident also in aquaculture species, with a median increase of 24% for growth-related traits and 22% for disease resistance traits (Houston et al., 2020). GS is thus likely to be a key technique for future breeding programmes of aquaculture species.

1.4.3 Genomic resources in large yellow croaker

1.4.3.1 Genome assemblies

High-quality genome assemblies provide opportunities to understand biological processes at the genome level for large yellow croaker. Six genome assemblies have

been released in the US-based National Center for Biotechnology Information (NCBI) service (**Table 2**), of which one assembly was from Daiqu strain, two were from Mindong strain and three were unknown.

Table 2. Current genome assemblies for large yellow croaker

Nr.	Name	Breed/strain	Total length (Mb)	Contigs	Contig N50	Assembly level
1	ASM74293v1	Donghai (Daiqu)	648.39	51,577	25,717	Scaffold
2	ASM435267v2	DH2-L1 (Mindong)	744.1	3,905	1,388,081	Chromosome
3	Larimichthys_crocea_chromosome_1.0	Unknown	689.17	9,930	130,633	Chromosome
4	LCFGL_HiC_1.0	Fufa I (Mindong)	721.26	1,576	2,833,482	Chromosome
5	ASM371158v2	Unknown	744.27	4,085	1,327,071	Chromosome
6	L_crocea_2.0	Unknown	657,94	16,979	277,487	Chromosome

1.4.3.2 Linkage mapping, QTL mapping and GWAS

A high-density linkage map is essential for the QTL mapping needed for MAS applications. At the same time, it is useful in order to provide a framework for whole genome assembly. To our knowledge, five linkage maps, all developed using only the Mindong strain, have been published (**Table 3**), in which some QTLs were identified linked to growth traits and *C. irritans* disease resistance.

Table 3. Summary of published linkage maps for large yellow croaker

Author & Year	Origin	Map length (cM)	Marker	Average interval (cM)
Ning et al.,2007	female	2959.1	181 AFLP+7 SSR	15.7
	male	2205.7	153 AFLP+8 SSR	13.7
Ye et al., 2014	consensus	1430.8	289 SSR	5.4
Ao et al., 2015	consensus	5451.3	10,150 SNP	0.54
Xiao et al., 2015	consensus	2632.0	3448 SNP	0.76
Kong et al., 2019	consensus	1885.6	5261 SNP	0.36

1.4.3.3 Genomic selection

GS technology has been applied to the selection of large yellow croaker for resistance to *C. irritans* (Zhao et al., 2021). The individuals with high GEBV in selection candidates were selected as broodstocks for the breeding of resistant strain, and the remaining individuals for the control strain. After 96-h, the survival rate of RS and CS were 59.2% and 9.9%, respectively. Furthermore, the 120-h survival rate of RS declined to 40.8%, while CS had no surviving individuals. The results confirmed that GS was an effective breeding technique for increasing the resistance against *C. irritans* in large yellow croaker.

The overall prospects of using these genetic tools in large yellow croaker are thus very good and promising, and the overall goal of paper III was thus to develop them for large yellow croaker.

1.5 The present PhD project, motivation, history and development

As laid out above, large yellow croaker is a new fish species with few or poorly developed selective breeding programmes. The overall objective of this PhD project was thus to provide basic knowledge in artificial fertilisation, then actually start up a new breeding programme for this important species, estimate genetic parameters, and measure genetic response, along the lines of what recently has been done for instance with river catfish in Vietnam (Nguyen et al, 2012, and Pham et al., 2020a,b,c).

However, the progress of the initial research project was not smooth, and some

unexpected difficulties and challenges appeared during the field experiments. As a result, changes had to be made, which also caused delays. Initially, wild stocks had been planned to be collected to establish the base population, generated by artificial fertilisation. This should include one wild stock from Mindong strain and one wild stock from the Daiqu strain. But no wild strains were captured. Even though a few fish were caught, they could be the farmed ones that escaped from nearby culturing cages. Thus, in the first year of the field experiment, only one farmed stock from the Mindong strain was utilised for generating new families and only 37 families were obtained due to challenging reproductive characteristics and unsuccessful artificial fertilisation. On top of that, all the families were lost in a typhoon before tagging. In the second year, two farmed stocks from the Mindong strain and Daiqu strain were utilised and 60 families were then obtained by improving the artificial fertilisation protocol. A random sample of 100 individuals per family was tagged, and the growth traits of body weight (BW), body length (BL) and body height (BH) were measured during tagging. These tagged fish were hereafter cultured communally in one large net cage. But again, due to a devastating typhoon in August the following year, all the tagged fish were lost. Thus, it was decided that we instead should start developing genomic resources, including SNP linkage maps by restriction-site associated DNA (RAD) sequencing. It was assumed, as genomic technology become more affordable, that the genomic resources based on genotyping-by-sequencing, especially the GS, will become more widely applied to increase genetic gain in the breeding programmes of large yellow croaker. Based on the finding in these three, rather distinctive, scientific disiplines, some suggestions will be given for designing an efficient and applied selective breeding programme for large yellow croaker.

2. AIMS OF THE THESIS

Large yellow croaker is a new aquaculture species, which only recently and insufficiently has been subject to contemporary selective genetic breeding. Three studies of this species are here presented, from reproduction suited for family construction, to genetic parameter estimation, and linkage map construction. The overall objective of the thesis has been to provide basic knowledge for designing an efficient and applied selective breeding programme to improve the growth traits of the fish. The specific goals of each paper are as follows:

- **Paper I:** To obtain high-quality gametes for artificial fertilisation in the selective breeding programme of large yellow croaker.
- **Paper II:** To estimate heritabilities and genetic and phenotypic correlations for growth traits based on crosses of Mindong strain and Daiqu strain, and establish a broad genetic base population for further selective breeding of large yellow croaker.
- **Paper III:** To construct consensus and parental linkage maps by doing a cross of the Mindong strain and Daiqu strain, to compare the linkage map to the latest physical map available (ASM435267v1), and to perform a QTL analysis and association analysis for growth traits.

3. GENERAL DISCUSSION

The domestication of large yellow croaker started in the middle of the 1980s. Now it is ranked as the top marine aquaculture species in China according to annual harvest production (China Fishery Statistical Yearbook, 2013-2020). However, the large yellow croaker industry has encountered great challenges, such as disease outbreaks, which have caused great economic loss and hampered the healthy and sustainable development of the industry. High quality strains with a fast growth rate and improved disease and stress resistance are urgently needed, achievable only by tedious and costly breeding programmes. Thus, this PhD project has attempted to establish a knowledge basis and important tools for running a contemporary selective programme for large yellow croaker, including controlled reproduction by artificial fertilisation, genetic parameters estimation for growth traits and linkage map construction (Paper I-III).

3.1 Artificial fertilisation and pedigree recording

Individual or phenotypic selection has been commonly used for commercial production of large yellow croaker since the first successful artificial breeding in 1985, mainly because of its simplicity. The accumulation of inbreeding might consequently be considerable, causing serious deterioration of economically important traits. Family-based selection is dependent on the ability to link individuals into family groups. Control of the reproductive cycle is one of the main limitations to obtaining family identification by separate rearing and also to be able to apply different mating designs in the breeding programme, together with increased investment and running costs (Duncan et al., 2013). Thus, a protocol for artificial fertilisation by gamete stripping is suggested for generating families of large yellow croaker in Paper I.

A notable trait of the family *Sciaenidae* is the ability to produce sound in the spawning season, suggesting that it might play a role in reproduction (Connaughton and Taylor, 1995), e.g. in the Japanese croaker (*Argyrosomus japonicus*) (Ueng et al., 2007). Based on personal observation, large yellow croaker could spawn naturally in large groups (>100 fish) after hormone therapy in commercial production, but were not mostly found to release eggs in smaller groups (<10 fish), which might be caused by lack of courtship and chasing in small groups. Thus, we did some initial experiments aiming

to determine the minimum size of groups that can produce croaking sound and courtship, and found that with a ratio of 2:1 (female to male) the minimum group size was 8 females and 4 males. Thus, in Paper I, 20 females and 10 males or 10 females and 5 males were used as a cohort of gametes striping to form social groups and courtship. The croaking sound and courtship were thus used to determine the egg stripping time in trial 2 of Paper I, instead of checking the more invasive way by a Pasteur pipette, which caused handling stress. The fertilisation rate and hatching rate in trial 2 were increased to 41% and 62%, respectively, by revising the doses and using croaking sound as an indicator for stripping. In comparison to Atlantic salmon, where the overall fertilisation rate was as high as 74% using cryopreserved milt and up to 81% using fresh milt (Kommisrud et al., 2020), the fertilisation rate in large yellow croaker is thus low, whereas with mass spawning, the hatching rate can be much higher, up to 90% (Yan and Wu, 1999). Therefore, although the reported fertilisation rate and hatching rate in paper I is an improvement, they are still not fully satisfactory to generate families on routine basis, since large numbers of family groups are required for reliable heritability estimations and to carry out a well-designed and efficient family-based selective breeding programme without severe inbreeding. Additional improvements of the protocol are thus still needed until satisfactory artificial fertilisation is achieved.

Pedigree recording is important in selective breeding programmes and hatchery management of aquaculture. Since physically tagging requires a certain size, typically minimum 5-10 g in the fish selective breeding (Gjedrem and Baranski, 2009), families need to be reared separately until tagging to obtain pedigree recording in the fish breeding programme, such as in the Norwegian salmon breeding programme started in 1971 (Gjedrem, 2010). Based on the improved artificial fertilisation protocol in Paper I, 60 families were obtained and reared separately until tagging. A random sample of 100 individuals per family was tagged with Passive Integrated Transponder (PIT) tags at six months of age, and thereafter reared communally. The main reason for delayed tagging in this study was that the typhoon season occurred earlier than expected this year and also an unexpected disease outbreak in the summer.

There are several drawbacks with physical tags. Due to separate rearing of fullsib families before tagging, common environmental effects may inflate heritability estimates, even with strict and standardised management. Special separate family

rearing units (i.e., tanks or hapas/nets) are also needed, imposing extra costs and practical challenges compared to standard nursery and rearing facilities.

Alternatively, parentage assignment by the use of DNA markers (microsatellite or SNP markers), which can be combined with mass spawning, makes it possible to reduce or eliminate the common environmental effects caused by individual fullsib family rearing, although some maternal effects may still remain, as shown in a study in Nile tilapia by Joshi et al. (2020). Since some broodfish are bound to have much larger genetic contributions than others in mass spawning of large yellow croaker (Liu et al., 2012), mass spawning of broodfish in small groups (>10 fish) should be applied to increase the effective population size. Moreover, mass spawning is labour-saving, and the stress of broodfish is much eliminated compared to artificial stripping. The estimation of genetic relationships by high-density SNPs has thus recently been used for pedigree reconstruction and genetic parameter estimation in large yellow croaker (Qiu et al., 2017). With the quick development of genotyping technologies, low cost parentage assignment will likely be increasingly used in large yellow croaker breeding programmes.

3.2 Heritability and genetic correlation

To plan a breeding programme, we need basic knowledge about genetic parameters, such as heritabilities and genetic correlations for the key commercial traits. The growth traits, i.e. BW, BL and BH, in large yellow croaker were thus measured during tagging, which means that the fish were only about 66 g on average, and the data was used for genetic parameter estimation for these growth traits in the Paper II. The heritabilities were medium to high: 0.31± 0.06 for BW, 0.33 ± 0.06 for BL and 0.41 ± 0.07 for BH. However, growth until harvest is a far more important economic trait for any breeding programme, and even higher heritability estimates for these traits were found in 22-month-old fish, at a size of 202 g for males (n=237) and 247 g for females (n=263): 0.63 ± 0.11 for BW, 0.60 ± 0.11 for BL, 0.53 ± 0.11 for BH (Qiu et al., 2017). However, we did not manage to calculate heritability for the harvest growth traits in the Paper II, since the tagged fish were lost in the typhoon.

Disregarding more advanced and costly schemes, with the use of genomic information and optimal contribution selection (OCS) (Skaarud et al., 2014; Houston et al., 2020), more simple programs, only considering growth as the main trait, can

sometimes be of interest in the initial phase of a breeding program. Using stochastic simulation, Gjøen and Gjerde (1998) found that phenotype selection produced higher genetic gain than BLUP for traits with $h^2 = 0.20$ and $h^2 = 0.40$, when the inbreeding rate was constrained to 1% per generation; i.e. only by manipulating the breeding design, not using OCS or quotas of candidates per family. Thus, individual selection for growth traits could be applied in the primary and small-scale breeding programme of large yellow croaker by using a sufficient number of breeders from different strains (i.e., Daiqu and Mindong strains) and restricting the number of contributing individuals per family to control the inbreeding level. However, restricting the number of contributing individuals per family requires control of family sizes, which is impossible to do with mass spawning.

Relatively high phenotypic and genetic correlations between BW, BL and BH were found in Paper II and in the study by Qiu et al. (2017), which indicate linked genetic influence, i.e., pleiotropy, or the non-random association of alleles resulting from linkage disequilibrium (Lynch and Walsh 1998). Joint genetic improvement for these traits thus could be accomplished by selecting only one trait. However, considering the phenotypic variation for each and the economic importance, body weight is suggested to be used as the best selection criterion of the three growth traits (BW, BH and BL).

3.3 Future trends: genomic solutions for large yellow croaker industry

Genotyping techniques, such as Genotyping-by-Sequencing (GBS) and restriction-site-associated DNA sequencing (RAD Seq), have facilitated the identification of large numbers of DNA markers randomly distributed throughout the genome. Together with the development of custom SNP arrays, these advances in genomics tools have also reached aquaculture species, permitting selection based on genomic information, such as MAS and GS (Meuwissen et al., 2001). MAS is an important strategy for traits controlled by major genes, but genetic linkage maps are a necessary framework for MAS breeding programmes. In Paper III, the consensus and sex-specific linkage maps were constructed for large yellow croaker, which is an important step towards locating QTLs accurately. In this study, we did not find any significant QTL for the growth traits investigated, which may be expected in such a small data material of only one family and

for these typically polygenic traits. Compared to linkage maps published previously, the present genetic map was based on the crossing of two strains instead of only one, as there are some genetic variations between the two strains (Lin et al., 2012). Furthermore, the Daiqu strain has later sexual maturation and better tolerance to lower temperatures than the Mindong strain (Liu and Mitcheson, 2008; Miao et al., 2014).

A significant advantage of MAS is that it is a way to select for traits that are difficult to measure on the selection candidates, such as disease resistance. In large yellow croaker farming, high quality strains that show better resistance to white spot disease, caused by the *C. irritans* bacteria, are required urgently by the industry. Thus, more studies should be focused on QTL detection and validation to develop strains that are resistant to serious diseases, enabling us to improve the traits continuously by breeding programmes based on GWAS and MAS. If such QTLs are found, the implementation or selection of them, may be different for the breeding nucleus from the lines distributed to the industry, as one would like to ensure a continued broad genetic base in the nucleus, allowing the QTLs to be recombined into new families, whereas a quick selection response, harvesting on the immediate profitable effects of the QTLs, should be emphasised in the lines used for production.

However, most of the QTLs detected are family-specific, with moderate effects, and the QTL with small effects may thus be missed in MAS (Sonesson, 2007). In contrast to MAS, GS can capture all genetic variances with genome-wide markers, even those with minor effects, and GS will thus be the most effective route to incorporating genotype data into long term selection decisions. GS is based on historical linkage disequilibrium (LD) in the population, and it both speeds up the selection process and increases the accuracy of estimates, compared to conventional pedigree-based estimates (Sonesson and Meuwissen, 2009). The routine implementation of GS is now largely carried out in salmonids like Atlantic salmon and rainbow trout (D'Agaro et al., 2021); and non-salmonids like Nile tilapia (Yáñez et al., 2020); not only for growth and fillet traits, but also for disease resistance and other quality traits (Houston et al., 2020). Also, in Atlantic salmon, a very high imputation accuracy was obtained by the use of genomic prediction, 0.89-0.97, depending on the density of the SNP sets ((0.5 , 5 K and 78 K) (Kijas et al., 2017). In rainbow trout, the genomic predictions outperformed the traditional EBV by 35% for fillet yield and 42% for fillet firmness using reduced density SNP panels (500 - 800) in a single-step genomic BLUP (ssGBLUP) model (Al-Tobasei et al., 2021). In Nile

tilapia, the accuracy of breeding value prediction using genomic data was up to 34% higher than using pedigree records for the trait feed efficiency (Barría et al., 2021) and up to 217% for resistance to Francisellosis (Joshi et al., 2021). These studies clearly highlight the huge potential of GS for wide range of traits in other fish species.

Indeed, GS has also been tried in large yellow croaker for resistance to *C. irritans*, where the survival rate of resistant strain was obviously higher than the control strain (Zhao et al., 2021). Genomic selection thus can improve selection programmes substantially, both for large yellow croaker and other new aquaculture species, especially with the development of low-cost genotyping techniques, such as RAD Seq .

3.4 A DNA-assisted breeding programme for large yellow croaker

Taking into account both the reproductive biology, operational costs, as well as breeding value estimation, a DNA-assisted breeding programme for large yellow croaker is suggested in **Figure 8**.

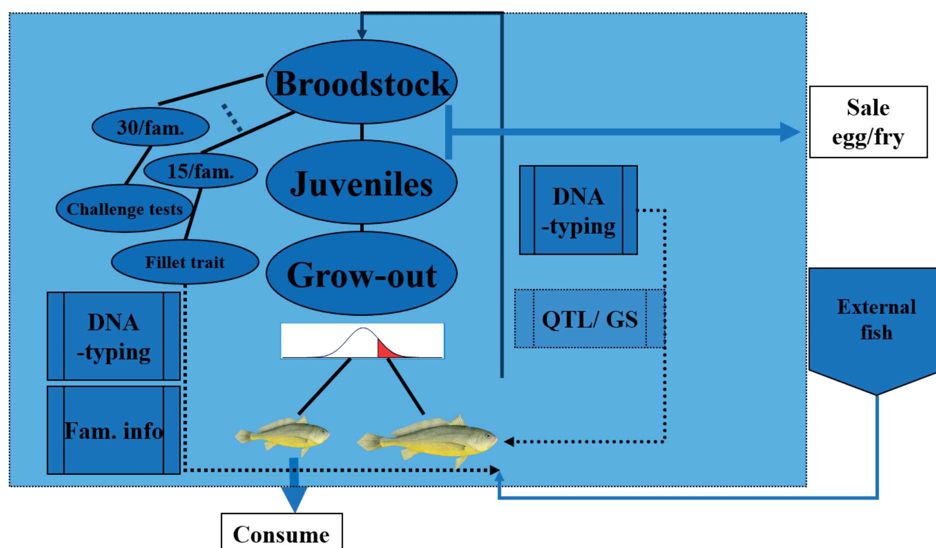

Figure 8. DNA-assisted breeding design for large yellow croaker (based on a design suggested by Hans Magnus Gjøen via simulation studies similar to Skaarud et al. (2011, 2014))

Selection from a broad base of broodstock is important in any breeding programme,

and since high quality broodstock is hard to find for large yellow croaker, this programme is an open synthetic population, as indicated by both an outflow and an inflow of fish from the system. This may indicate that fish from the programme which have survived a natural outbreak of a severe disease, could be introduced back into the system after quarantine, or fish from another farm could be introduced to broaden the genetic variation.

A sample of 30 fish from each family is used for challenge testing, such as for low temperature or disease resistance, and 15 or more fish from each family are used for measuring invasive quality or fillet traits. This ensures an even representation of all families when BVs are being estimated based on all registered phenotypes and marker information through MAS and GS. In addition, the design allows a very strong selection for the important economic trait, growth, which can easily be monitored and selected for in a huge number of individuals participating in the large grow-out group.

The largest benefit of the design is that it is flexible, both with respect to family size, which can vary greatly for marine species, and utilisation of internal and external broodstock, which would be beneficial in many marine species due to lack of sufficient good quality broodstock. This flexibility also will be crucial if calamities, like a typhoon, hit again, as it allows for simple and distributed growing of the fish, serving as backups. Finally, the design utilises the large within-family variation, which accounts for half the genetic variation and is not well utilised in conventional aquaculture breeding programmes (Sonesson and Meuwissen, 2009).

4. CONCLUSIONS

The main findings of the thesis are:

- A revised protocol for artificial fertilisation is suggested for generating families in selective breeding programmes for large yellow croaker. The main points are selecting broodstocks at the same age and size, performing double hormone injections, and determining stripping time by observing courtship behaviour to reduce handling-stress.
- Moderate heritabilities (0.31-0.41) were found for BW, BL and BH of large yellow croaker at the age of six months. Genetic and phenotypic correlations among the growth traits were all positive and high. This establishes a potential to select for growth-related traits in large yellow croaker.
- Consensus and parental linkage maps were constructed. The consensus genetic linkage map offers a larger number of markers representing Daiqu and Mindong strains. The map was adjusted based on the physical map to generate the integrated consensus and sex-specific linkage maps. Higher recombination rates were found in the integrated female map, compared to the integrated male map. The study indicates that there may be genetic differences between the two strains Daiqu and Mindong, which may have implications for breeding programmes using DNA-information in a future selection scheme.

5. CHALLENGES AND RECOMMENDATIONS

Based on my work experience and the process with this PhD project, the practical implementation of modern selective breeding for large yellow croaker has several challenges. The recommendations given here are thus aiming at implementing the breeding programme successfully and sustainably:

- *Manual phenotyping*, or recording of traits, is very labour-intensive, and in some cases biased due to human errors. For example, the tagging and recording of growth traits for the Paper II were performed in a small cottage made of wood, floating and moving strongly on the sea, making correct weighing challenging. The automation of phenotyping should thus be developed, i.e., automating weighing system for BW and image-based approaches for BL and BH. Then, the accuracy and repeatability of phenotypes of large sample sizes will be improved, while fish welfare will also be enhanced by reducing fish handling.
- *The risk associated with live feed (e.g. copepods) for fry production.*
  The availability of captured copepods from the sea is unstable, and there is always a risk of transferring pathogens to the vulnerable fry with the live feed. Thus, live feed culturing techniques, especially copepods, are therefore necessary to ensure the feed security in a pathogen-free environment and to improve feed sustainability.
- *Unsustainable financial and technical support.*
  Genetic research and breeding programmes require significant financial support. A long-term breeding programme will be vulnerable and discontinued once the support withdraws, caused by the absence of a formal and responsible breeding society or organisation. A better market link should be established to ensure returns on investment and to strengthen the financial support from cooperatives and private companies. Fish farmers' opinions should be included by incorporation of their feedback in the research decisions.
- *Poor infrastructure and facilities for aquaculture.*
  These infrastructure and facilities are easily destroyed by typhoons occurring frequently in the summer season, as illustrated by the loss of the families generated by artificial fertilisation in my research. The infrastructure and facilities for aquaculture should be advanced and modernised.

- *Coordination among different researcher groups* is required to avoid repeated work and wasted resources. Collaborations among research institutions, aquaculture companies and farmers should be promoted to exchange genetic materials and to develop a genetic resource network.
- *Lack of trained aquaculture geneticists in the commercial sector*.
  Regional and national training centres in genetics and breeding should be established for researchers to ensure that genetic research and genetic material development are appropriate for the commercial sector, applied properly and disseminated efficiently to achieve maximum benefit.
- *The legal framework and intellectual property issues* for the use and exchange of genetic materials and data should be strengthened.
- *Genetic diversity and inbreeding.*
  If the genetic basis of the breeding nucleus is sufficiently wide, no wild large yellow croaker should be incorporated in the breeding programme in the future. To maintain a sustainable breeding programme, the inbreeding must be controlled by keeping a sufficiently high number of families and constraining the number of selected individuals per family, or by using Optimal Contribution procedures (Skaarud et al., 2011 & 2014). To optimise the use of resources and manage genetic variation sustainably, the optimal number of families is as a rule of thumb at least 300 in fish-breeding schemes with multiple traits, as suggested by Skaarud et al. (2014) based on a simulation study.

6. FURTHER WORK

Genetic improvement is an ongoing process with tremendous opportunities. Given the results presented in this thesis, the following points are suggested for further research work.

- More traits, such as harvest weight, specific disease resistance and cold tolerance, are likely to be included in the breeding goal of a future breeding programme.
- Multiple segregating families should be used to understand the SNP allelic diversity, and QTL mapping for specific disease resistance should also be performed.
- Given the availability of several genetic maps and two genome assemblies for large yellow croaker, some efforts should be made to compare and integrate these data with the goal of validating, improving and integrating the assemblies and generating a convention for LG nomenclature to avoid confusion.
- High-density SNP genotyping arrays should be developed for MAS and GS.

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Paper I

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SHORT COMMUNICATION

Artificial fertilization and generating families for a selective breeding programme of large yellow croaker (*Larimichthys crocea*)

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Abstract Large yellow croaker is an important marine aquaculture species in China. The aim was to determine an appropriate protocol of artificial fertilization for family construction in the breeding programme based on two trials. In trial 1, luteinizing hormone-releasing hormone A_3 ($LHRHA_3$) was injected once, with a dosage of 2 μg/kg for females and 1 μg/kg for males. The latency time was in the range of 30–34 h. The maturation stage was checked by extracting a few eggs with a Pasteur pipette. The fertilization rate and hatching rate were 27 and 52%, respectively. The percentage of females with spawning difficulties was 30%. In trial 2, the females were injected $LHRHA_3$ twice: with a first dose of 0.8 μg/kg and a second dose of 2 μg/kg, at an interval of 10 h, whereas the males were still injected once. The latency time was in the range of 29.5–35 h, determined by only observing courtship behaviour of males. The females with spawning difficulties decreased to 10%, and the fertilization rate and hatching rate also improved to 41 and 62%, respectively.

Keywords Large yellow croaker · Artificial fertilization · Hormone injections · Latency time

Introduction

Large yellow croaker (*Larimichthys crocea)* is a batch spawner that naturally occurs in temperate seawater regions of East Asia. The aquaculture production was near 1.5×10^5 tons in 2015, accounting for 10% of the cultured marine fish production in China (Tang 2016), and now it is the largest marine aquaculture fish species in this country. However, the production is still based on strains that have not been subjected to a modern breeding programme which is crucial for further development of the aquaculture industry. A basic requirement for implementing a family-based sustainable breeding programme for a new fish species is the knowledge of control of reproduction to establish enough full- and halfsib families. Natural spawning at first glance seems to be a good choice to generate families, since large yellow croaker is sensitive to stress caused by handling (Duan et al. 2001). By natural spawning, a certain number of females and males are kept in a tank, and the species reproduce naturally. The consequence of this approach is that full- and halfsib families are mixed in

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the tank, and DNA-typing is required to obtain pedigree information; this will introduce a certain cost when a relatively large number of fishes need to get parental assignment. Moreover, since sperm competition gives unequal contributions from males, some broodfish are bound to have much larger genetic contributions than others (Liu et al. 2012). Thus, a relatively large number of non-contributing broodfish will inevitably reduce the effective population size (N_e) and increase the inbreeding rate. Single pair mating by natural spawning was also tested in some initial trials, but it was not successful; most likely because positive social interaction signals were lacking for the initiation of sexual maturation.

Applying artificial fertilization is an alternative way to establish full- and halfsib families for the breeding programme, but high-quality gametes are critical for successful fertilization. Post-ovulation oocyte ageing in the ovary of fish is one of the limiting factors for successful artificial fertilization (Bahre Kazemi et al. 2010). Thus, it is especially important to know the latency time, the time interval between hormone injection and stripping, when conducting artificial fertilization. The sperm-quality is easier to control compared to eggs. For instance, it was reported that very good-quality sperm can be obtained during the reproductive period, even without hormone induction in pikeperch (Zakes and Demska-Zakes 2005). However, sea water should be avoided during milt stripping, as the sperm motility will be activated by water and the duration of motility will last for only a short period for most fish species (Cosson 2004). With sperm energy storage exhausted after mobilization, the ability to fertilize eggs will quickly be lost consequently.

For cultured large yellow croaker, it is essential to determine latency time to avoid egg over-ripening in artificial fertilization. Moreover, in the breeding programme, spawning time should be synchronized to make full- and halfsib families as uniform as possible, both in size and age, and to reduce seasonal or temporal environmental effects on families. It was previously reported that the latency time for large yellow croakers was about 48 h with a dose of 1–2 μg/kg $LHRHA_3$ at a water temperature of 22–24 °C (Zheng et al. 2006). This reported latency time was initially tested under similar conditions, but the over-ripening problem was found to be very severe.

Thus, the aim of this study was to find an appropriate way to determine the best time for egg stripping after ovulation, and to establish an artificial fertilization protocol for large yellow croaker, as a preparation for a future selective breeding programme.

Materials and methods

Trial 1

Broodstock managements

In March 2013, 150 two-year-old large yellow croakers (sex ratio♀:♂ = 2:1), from the Mindong strain, were selected as broodstock from a farm in Fuding, Fujian province. All the selected candidates were in good health condition, with no body wounds. They were reared in a tank (6 m × 8 m × 1.2 m), with 80% daily water exchange, and were fed on minced fresh mackerels mixed with complex vitamins and minerals additives, twice a day, at a ratio of 5% of body mass per day. One month before mating, the broodstock were sorted by sex and then reared in separate tanks; making each group consist of 20 females and 10 males. Males were identified by the appearance of milt when the abdomens were pressed gently.

Hormone stimulation

Before hormone injection, each group of fish (20 females and 10 males) were anaesthetized with MS-222 (30–40 ppm) to minimize stress. This treatment took place at 06:00 in the morning, and the dosage of $LHRHA_3$ injection was 2 μg/kg for females and 1 μg/kg for males. Treated fish were placed in a breeding tank with clean seawater (24 °C) and aeration. Then the tank was covered with black plastic sheets to obtain a dark environment. All the lamps were also turned off in the incubation room and noises were minimized. Females were checked every 2 h from 26 to 36 h post injection, i.e. from 08:00 to 18:00 the next day. Females with large bellies were gently caught for closer examination. If the abdomen was soft, a few eggs were checked from the genital pore by a Pasteur pipette with a long tip. When the eggs were spherical, translucent and slick,

rather than collapsed and flaccid, it was considered to be the right moment to strip eggs. A few females were selected randomly for dissections to visualize the ovaries status, both before injection and after injection.

Gametes collection and artificial fertilization

Ready to spawn females and males were again anesthetized with MS-222 (30–40 ppm), and washed with clean seawater, wrapped in wet towels and the genital openings were dried off. The females were then stripped by gently stroking of the abdomens, and eggs from each female were collected in a separate 1000 ml beaker. Milt was collected into a 1 ml syringe, with gentle pressure to the male's abdomen, and then kept on ice in a polystyrene box (4 °C) until fertilization. During stripping, it was ensured that no water or urine contaminated the gametes. To generate paternal halfsibs and fullsibs, the milt from one male was used to fertilize eggs from two females. After stripping, these fish were put into a well-aerated tank, containing 200 L clean sea water, for recovery. Forty grams of eggs (800 eggs/g) and 0.2 ml (1.45×10^{10} sperms/ml) milt were placed in the same beaker, without adding any water. They were mixed with a sterilized feather for 40 s, and then 1 L clean seawater (24 °C) was added gradually and stirred with the feather at the same time. After 5 min of incubation, two layers of eggs appeared. The buoyant ones were collected, rinsed and then transferred to 1 m^3 breeding cylinders with 24 °C seawater (Fig. 1a) for further incubation, whereas sinking and white ones were considered as non-viable and thrown away. The average hatching time was 25 h.

On the second day after hatching, rotifers were put into the breeding buckets as live weaning feed for the larvae, on day six artemia were added, and on day 12, live copepods were added. The hatching and start-feeding buckets had 30% water exchange every day. Fifty days after hatching, the fingerlings (5 ± 1.1 cm in total length, Fig. 1c) were transferred to the net cages in the sea and each family cultured separately until tagging.

Trial 2

In March 2014, two-year-old broodstocks from the Daiqu strain (90 fish from a farm in Xiangshan, Zhejiang province) and the Mindong strain (90 fish from a farm in Fuding, Fujian province) were selected with a sex ratio of 2:1(♀:♂). The selection criteria were the same as in trial 1. The two strains were crossed, and paternal half- and fullsib groups generated. These fish were grouped into two types of mating cohorts: one including 10 Daiqu females and 5 Mindong males (six cohorts) and the other including 10 Mindong females and 5 Daiqu males (six cohorts). Based on the evaluation of trial 1, some adjustments were made to the breeding protocol:

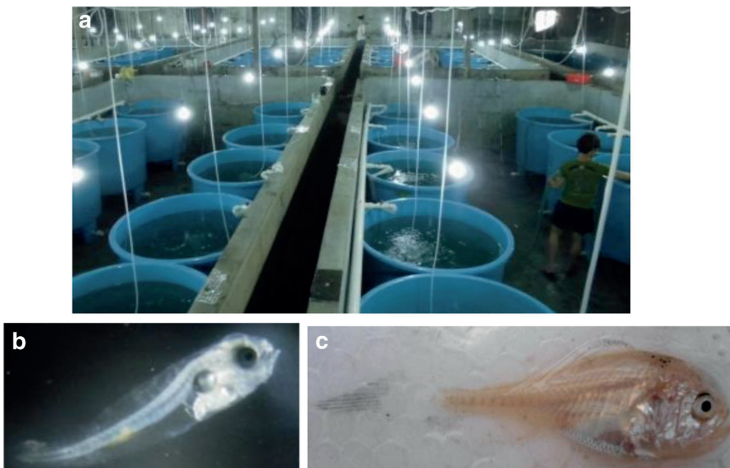

Fig. 1 **a** Buckets for incubation; **b** larvae at hatching; **c** fingerlings, 50 days after fertilization

Double injections

The females in this trial were injected twice, at an interval of 10 h. The first injection was given at 6:00 in the morning, with a dosage of 0.8 μg/kg, and the second treatment was given at 16:00 in the afternoon, with a dosage of 2 μg/kg. The males were still only injected once, at the same time as the second injection of the female, with the same dosage as in trial 1, i.e. 1 μg/kg.

Courtship behaviour observation

In order to stimulate social signals for sexual maturation, the treated fish were placed in cohorts, as described above. The behaviour of the broodfish was monitored every half an hour from 28 h post injection, but without invasive checking by a Pasteur pipette. The time was recorded as latency time when males' courtship behaviour appeared; i.e. males chased females and emitted repetitive throbbing or drumming sounds by vibration of the sonic muscles near the swimming bladder, thereby the name croaker. Then artificial fertilization was performed in the same manner as in trial 1.

Fertilization rate and hatching rate of the two trials

The fertilization rate and hatching rate were calculated from six randomly sampled fullsib families. The fertilization rate was first calculated by observation of 100 randomly fetched buoyant eggs 2 h after fertilization, examined under a microscope. At this time, the development of normal eggs had reached the stage of 32-cell-division, which was possible to identify in the fertilized eggs. Then the fertilization rate was converted to be based on 100 total stripped eggs by the ratio of buoyant and sinking eggs. The hatching rate was determined as the proportion of hatched larvae to 100 fertilized eggs. Fertilization and hatching rates of the two trials were compared using a *t* test.

$$\text{Fertilization rate} = \frac{\text{Number of fertilized eggs}}{\text{Total number of stripped eggs}} \times 100\%$$

$$\text{Hatching rate} = \frac{\text{Number of hatched larvae}}{\text{100 fertilized eggs}} \times 100\%$$

Results

In trial 1, the latency time for females was 30–34 h, using a single $LHRHA_3$ injection and by Pasteur pipette checking, in 23.6 °C seawater. In trial 2, the latency time was 29.5–35 h, using double injections and by courtship behaviour observations, in 23.7 °C seawater. The fertilization rates of the two trials were 27.5 ± 2.1 and 41.4 ± 2.3%, respectively; significantly different ($p < 0.05$). The hatching rates of the two trials were 52.3 ± 4.8 and 61.5 ± 8.7%, respectively; not significantly different ($p > 0.05$) (Table 1).

Before injections, the females ready to spawn had pink genital papilla (Fig. 2a). The white and tiny eggs in the ovary were connected with each other like one cohesive substance (Fig. 2b). After injections, most females had large bellies and soft abdomens. These females could be stripped easily and ovulated eggs were separable and transparent (Fig. 2c), but there were still some white immature eggs left in the ovaries.

Also, eggs in some females were found hard to strip even after full latency time, both in trial 1 and trial 2. Despite extremely large bellies, the abdomens did not soften. The eggs inside were white and still connected with each other, although some eggs' colour changed from white to semi-transparency (Fig. 2d). These females had spawning difficulties and were impossible to strip. In trial 1, the percentage of these poorly

Table 1 Fertilization rates and hatching rates

Trials	Water temperature ± SD (°C)	Latency time range (h)	Fertilization rate (%) ± SD	Hatching rate (%) ± SD
1	23.6 ± 0.16	30–34	27.5 ± 2.1	52.3 ± 4.8
2	23.7 ± 0.14	29.5–35	41.4 ± 2.3	61.5 ± 8.7

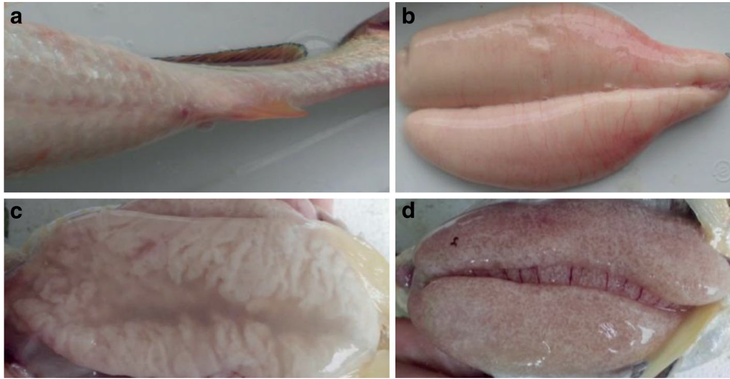

Fig. 2 **a** Pink genital papilla of females; **b** immature eggs in the ovaries, before hormone injection; **c** mature eggs, ready to be stripped; **d** non-mature eggs after injection that could not be stripped easily, even after expected latency time

matured females was about 30%, whereas in trial 2 it decreased to about 10%, both in the Mindong and the Daiqu strain. Eventually, 37 families were generated with 150 broodfish tested in trial 1, whereas 60 families were obtained with 180 broodfish tested in trial 2.

Discussion

For cultured large yellow croakers, hormone stimulation is necessary to induce final oocyte maturation and synchronize the ovulation. $LHRHA_3$ (a GnRH analogue) can have a stimulating effect on pituitary gonadotrophs and consequently increase the secretion of gonadotropins. In trial 1, $LHRHA_3$ was injected once, but there were still 30% poorly matured females whose eggs were hard to strip, and the fertilization rate was low. In trial 2, using double injections, the poorly matured females decreased to 10%, and the fertilization rate increased significantly. The hatching rate was also improved, although not significantly. The first injection was done to promote maturation of the eggs and the second one was to induce the release of eggs from the ovary (Shan Jian 1985). For Senegalese sole (*Solea senegalensis*), a repeated GnRHa injection protocol was also more effective than a single injection for stimulating egg production by stripping, without compromising egg quality (Rasines et al. 2012). Double injections with $LHRHA_3$ are thus recommended for large yellow croaker, especially for the less matured parent fish, where it can substantially accelerate the maturation of the eggs. The hatching rate could be affected partly by herterosis effect from crossing of two strains in trial 2.

Other spawning hormones may also be evaluated for more successful spawning, e.g. human chorionic gonadotropin (hCG). In pikeperch, the highest percentage of ovulated females was obtained in the group stimulated with hCG, giving up to 100% ovulation, and the development of the oocytes in this group was also more rapid than in the group stimulated with only GnRH analogue (Zakes and Demska-Zakes 2005).

The latency time is very important to determine the optimal egg-stripping time, and a significant determinant for the egg quality (Craik and Harvey 1984). If egg stripping is done much later than optimal latency time, ageing of ovulated eggs in the ovary or the coelomic cavity occurs, causing egg over-ripening, which is associated with a decrease in egg viability. In rainbow trout (*Oncorhynchus mykiss*), biochemical and histological changes occur inside the eggs and in the ovarian fluid during over-ripening (Lahnsteiner 2000), and the egg viability significantly decreased with the egg protein fragments being accumulated in the ovarian fluid (Hélène et al. 2004). The duration time of ovulated egg viability in the fish ovarian fluid seems to be temperature dependent. For example, cold water fish species like rainbow trout (*Salmo gairdneri* R.) can hold their eggs in the ovaries for 4–6 days post-ovulation, at about 10 °C, without decrease in viability (Springate et al. 1984), and Atlantic salmon eggs were still of good quality even for one week after ovulation at 9.1 °C (de Gaudemar and Beall 1998). In contrast, in warm-water fish species, eggs deteriorate rapidly after ovulation. For instance, the maximum period between ovulation and the deterioration for striped bass (*Morone*

saxatilis) was only 15–30 min (Rottmann and Chapman 1991). As large yellow croaker is a warm-water species (18–25 °C), the duration time of ovulated egg viability could be very short, and the latency time between hormone injection and stripping is thus critical to avoid egg over-ripening. The previous report only mentioned the latency time, but the measuring method was not given (Zheng et al. 2006). In this study, the latency time is much shorter and the over-ripening problem is improved by observing courtship behaviours.

Most females of large yellow croaker could be stripped easily, and good-quality eggs were obtained when the correct latency time was applied, but there were still some immature white eggs left in the ovaries. The reason could be that large yellow croaker is a batch spawner. Monitoring method may be another factor that affected eggs' maturation processes. In trial 1, monitoring by a long tip Pasteur pipette was a too invasive method, which caused the fish to become overly nervous and stressful.

The handling stress likely induces cortisol synthesis, which could affect reproductive characteristics of the fish by altering gonadal steroids levels through the hypothalamus–pituitary–gonadal axis. In jundia (*Rhamdia quelen*), handling stress of mature females resulted in higher cortisol level, but lower 17β-estradiol level, compared to the control group; fewer oocytes could be stripped from the stressed fish, and the quality of these eggs appeared reduced (Soso et al. 2008). Stress during reproductive development also delayed the ovulation and reduced the quality of gametes in rainbow trout (Campbell et al. 1992). Large yellow croaker is a stress-sensitive fish, and the maturation and ovulation of oocytes might be stopped as a reaction to the handling stress by Pasteur pipette checking applied in trial 1. Consequently, the higher occurrences of females' spawning difficulties could be due to the successive checking and handling before complete egg maturation. In trial 2, the latency time was determined by observing courtship behaviour and less spawning difficulties occurred. More non-invasive methods should thus be tested for measuring the stages of reproductive maturation in the stress-sensitive fish, such as measurement of sex steroid hormones in surface mucus and use of ultrasound imaging for monitoring gonadal development (Schulz et al. 2005; Novelo and Tiersch 2016).

Conclusion

From these two trials of artificial fertilization in large yellow croaker, we generated 37 and 60 families, respectively. Although the number of tested families in each generation was a bit low for an efficient breeding programme, the suggested protocol would still be helpful for family construction by artificial fertilization in a future selective breeding programme. The main points were to select broodfish at the same age and size, perform double hormone injections and keep handling of the fish at a minimum level. However, there are still additional improvements of the protocol before satisfactory artificial fertilization is achieved, such as routines to further minimize the handling stress.

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Compliance with ethical standards

Ethics statement This study was approved by the Institutional Animal Care and Use Committee (IACUC) of Zhejiang Ocean University, Zhoushan, China, and all persons involved in fish handling had special training before conducting the relevant experiments.

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Phenotypic and Genetic Parameter Estimation for Growth Traits in Juvenile Large Yellow Croaker (*Larimichthys crocea*)

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Paper II

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Research Article

Phenotypic and Genetic Parameter Estimation for Growth Traits in Juvenile Large Yellow Croaker (*Larimichthys crocea*)

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ABSTRACT

Heritabilities, as well as genetic and phenotypic correlations, were estimated for body weights (BW), body lengths (BL) and body heights (BH) in large yellow croaker, *Larimichthys crocea*. By crossing the Mindong and Daiqu strain, 60 fullsib families (offspring of 32 males and 60 females) were generated and reared separately. Environmental conditions of each family were standardised, and the body traits were recorded at six months of age. Heritabilities for the body traits were medium to high: 0.31 ± 0.06 for BW, 0.33 ± 0.06 for BL and 0.41 ± 0.07 for BH. The correlations among the three growth traits were positive and high in all cases, with the phenotypic correlations ranging from 0.84 to 0.91 and the genetic correlations ranging from 0.74 to 0.95. The results indicate that the growth traits of juvenile large yellow croaker could be improved efficiently by selection in a future breeding program.

Keywords: Large yellow croaker; Daiqu and Mindong strains; Growth traits; Genetic parameters

INTRODUCTION

The marine fish species large yellow croaker (*Larimichthys crocea*) is occurring in the coastal regions of East Asia and are generally found in temperate seawaters (18-25°C). The wild populations have been severely depleted due to overfishing and environment deterioration [1]. The culturing of the species has been successful since the late 1980s, and it is now one of the most important marine aquaculture fish species in China, with a total production of about 180,000 tons in 2017; accounting for more than 12% of the marine fish aquaculture production [2]. Large yellow croaker is a good food resource with excellent nutritional value, providing high quality protein and the healthy omega-3 fatty acids, including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). The swim bladders are also a popular functional food [3], and the market demand for high quality large yellow croaker is increasing.

Since the 1980s, large yellow croaker has been cultured and to some extent been subject to phenotypic selection for increased growth, but without pedigree information and control of inbreeding. Genetic diversity of cultured croaker has thus significantly been reduced compared to the wild populations [4], and the quality has declined for economically important traits, especially growth and disease resistance. An efficient breeding program for large yellow croaker is thus required.

At the beginning of most fish breeding programs, growth is often targeted as the main trait, since it is easy to record and increased growth rate generally improves the profitability of the production. And considerable selection potential for growth has been demonstrated in selective breeding programs of several important aquaculture fish species. For example in Atlantic salmon (*Salmo salar*), the genetic change per generation was up to 15% in the first generations of the Norwegian selective breeding program [5]; and correspondingly, sustained genetic gain for growth was also high in Nile tilapia (*Oreochromis niloticus*), with 10–15% per generation in the genetically improved farmed tilapia (GIFT) project [6]. Compared to farm animals, realised genetic responses for increased growth rate are larger in aquatic species [7]. The economic potential of starting a family-based breeding program for increased growth in large yellow croaker should thus be considerable.

Planning of optimal selective breeding requires knowledge of heritabilities and genetic correlations, which are crucial to calculate unbiased breeding values and to predict expected genetic progress. To date, three studies of the genetic parameters for growth traits in large yellow croaker have been reported, but based on the Mindong strain only and with relatively small sample sizes [8-10]. There also exists another geographically and genetically different strain known as the Daiqu strain in the north region of East China Sea [1], whose genetic diversity and growth rate were found to be higher than the Mindong strain [11,12]. Currently, no heritabilities or genetic correlations for growth traits based on crossing of the

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two strains have been reported. The specific aim of this study was to obtain estimates of these genetic parameters for BW, BL and BH based on crossing individuals from the two strains.

MATERIALS AND METHODS

Broodstock fish and design of experiment

Ninety sexually mature fish of Daiqu strain were selected from approximate 1000 individuals at an aquaculture farm in Xiangshan, Zhejiang province (29°5'N, 121°8'E) , and correspondingly, ninety sexually mature fish of Mindong strain were selected from approximate 1000 individuals at another farm in Fuding, Fujian province (27°3'N, 120°25'E). Both strains were sampled with a sex ratio of 2:1 (♀:♂). In March 2014, the broodstock of both strains were transported to the Zhejiang Dahaiyang Science and Technology Co. Ltd. at a sea-site location in Mazhan, Cangnan, Zhejiang province. The mean body weight was 670 ± 123g (mean ± SD) for the Daiqu strain and 500 ± 108g for the Mindong strains. All fish were dorsally tagged with Passive Integrated Transponder (PIT) tags (12 mm × 2.12 mm and 0.09 g). The two strains were kept in two separate tanks (6 m × 8 m × 1.2 m) and fed twice a day with minced fresh mackerels mixed with complex vitamins and mineral additives at a ratio of 5% of body mass per day.

One month later, the brood fish were injected with luteinising hormone-releasing hormone A_3 (LHRH A_3), with a dose of 2 μg/kilo for females and 1 μg/kilo for males. This resulted in an observed spawning time in the range of 30-34 h post injection [13], when milts and eggs were collected by hand-stripping. A nested design was applied for mating: one Mindong male mated with two Daiqu females, or one Daiqu male mated with two Mindong females. Finally, 32 sires (14 from Daiqu and 18 from Mindong) and 60 dams (27 from Daiqu and 33 from Mindong) produced 60 crossbred fullsib families from the two strains, i.e. comprising 27 fullsib families from Daiqu♂ × Mindong♀-cross and 33 fullsib families from Mindong♂ × Daiqu♀-cross [13].

The culturing of fullsib families

After fertilisation, a standardised quantity of 20 ± 0.2 g floating eggs per family were transferred to separate 1 m^3 breeding cylinders for incubation. After hatching, the larvae were fed with rotifers, artemia and copepods, successively. The seawater condition was standardised, with salinity at 24.2 ± 0.1%, temperature at 23.7 ± 0.1°C, dissolved oxygen level at 5-7 mg/L and pH at approximately 8. The water exchange rate was set to 30% per day.

After 50 days, when the average total length of fingerlings was 5 cm, about 1000 randomly sampled fingerlings per fullsib family were transferred to separate small net cages (1 m × 1 m × 2 m) in the sea for on-growing until tagging. They were fed twice a day with commercial fish pellets at a ratio of 5% of body mass (52-55% fish protein powder, 2% fish oil, Shanghai Nonghao Co. Ltd.).

Data collection

In September 2014, when the fish were 6 months old, a random sample of 100 individuals per family (total 6000) were anesthetised with MS-222 (20-30 ppm) after starvation for two days, and body weight, body length (from tip of snout to the last vertebra) and body height (in front of the first ray of dorsal fin from the dorsal margin of the body to the ventral margin) were measured (Figure 1). Afterwards, they were tagged with a PIT tag in the abdominal cavity (Figure 2). The measuring and tagging time per fish was about one minute. If the tagged fish did not recover within five minutes, it was replaced by another fish from the same family.

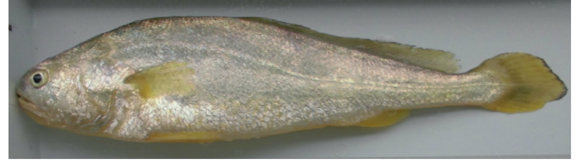

Figure 1: Body measurements taken on large yellow croaker: BL and BH.

Figure 2: PIT tagging fish with hand-held injection device.

The tagged fish were hereafter cultured communally in one larger net cage (3 m × 3 m × 4 m). The fish were intended for further growth and later selection and mating, but due to a devastating typhoon in August the following year, all the tagged fish were lost. The results reported here are thus based on the data recorded at tagging, i.e. at six months of age.

Statistical analysis

The estimations of heritability and correlations between body weight, body length and body height were achieved with a multi-trait animal model, using the ASReml software (version 4.1) [14]. The following model was used, in matrix notation:

$$y = Xb + Z_1c + Z_2a + e$$

Where, **y** is a vector of individual observations, partitioned for each growth trait, **b** is a vector of fixed effects (i.e. the Daiqu or Mindong strains and 14 recording dates), **a** is a vector of random additive genetic effects (number of individuals=6000), **c** is a vector of common environmental effects pertaining to fullsibs (60 families) and **e** is a vector of individual random error effects. **X, Z_1** and **Z_2** are known design matrices assigning observations to levels of **b, c** and **a**, respectively. The heritability was calculated as $h^2 = \frac{\sigma_a^2}{\sigma_p^2}$, where σ_a^2 is the additive variance of each trait and σ_a^2 is the phenotypic variance of each trait.

The additive genetic correlations between traits, *i* and *j*, was calculated as $r_a = \frac{cov_{ai \cdot aj}}{\sigma_{ai}\sigma_{aj}}$ and the phenotypic correlations as $r_p = \frac{cov_{pi \cdot pj}}{\sigma_{pi}\sigma_{pj}}$, where $cov_{i,j}$ is the covariance and σ_i, σ_j are standard deviations of the two traits, i.e. for the additive genetic (a) or phenotypic (p) effects, respectively.

RESULTS

Growth traits, i.e. BW, BL and BH, of 6000 six-month old individuals from 32 sires and 60 dams were measured at tagging.

Descriptive statistics for the three traits are given in Table 1. Twenty-three possible outliers were noted by ASReml for each trait but were still found to be plausible observations after individual checking. The relative variation, quantified by the coefficient of variation (CV), was high for BW but relatively low for BL and BH.

Genetic parameters for the three growth traits are presented in Table 2. Heritabilities for all the three growth traits were high, ranging from 0.31 (BW) to 0.41 (BH). The phenotypic and genetic correlations among these growth traits were also relatively high. The highest phenotypic (0.91) and genetic correlation (0.95) was between BW and BL, while the lowest correlations were between BL and BH (r_p=0.84, r_g=0.74). The genetic correlation between BW and BL was higher than the phenotypic correlation, while the opposite was found for the other traits. The fullsib family variance component, associated with the common environment effect, was not estimable in the present dataset and was set to zero.

Table 1: Means, standard deviations (SD) and coefficients of variation (CV) of body weight, body length and body height at 6 months' age of the F1 generation (N=6000).

	Mean	SD	CV (%)
Body weight(g)	66	22	33
Body length (cm)	15.6	1.7	11
Body height(cm)	4.2	0.6	13

Table 2: Heritability (diagonal, in bold), genetic (above diagonal) and phenotypic (below diagonal) correlations with standard errors (±SE) between body weight, body length and body height.

	Body weight	Body length	Body height
Body weight	0.31 ± 0.06	0.95 ± 0.02	0.79 ± 0.06
Body length	0.91 ± 0.004	0.34 ± 0.06	0.74 ± 0.07
Body height	0.87 ± 0.01	0.84 ± 0.01	0.41 ± 0.07

DISCUSSION

The heritabilities for the measured growth traits of juvenile large yellow croaker ranged from 0.31 to 0.41 in the present study, suggesting that rapid genetic gains could be achieved by BLUP selection for these traits in juvenile fish. Generally, selection should be made close to market size of the fish, but due to loss of all tagged fish in the typhoon season, heritability for growth at harvest was not available in this study. Liu et al. [8] estimated heritabilities for growth traits of 20 month old fish in the range of 0.09-0.19 based on 599 individuals, but based only on two fullsib/halfsib groups (4 males × 4 females and 4 males × 3 females); and in another study [9] in 13-month old fish the estimates ranged from 0.02 to 0.36, based on 959 offspring from only 10 dams and 15 sires; whereas Dong et al. [10] estimated heritabilities for BW and BL to be 0.60 and 0.59, respectively, using genomic prediction based on 500 two-year old fish. Heritabilities for growth traits will often vary with age in fish; for instance, higher heritabilities for body weight and total length were found at 270 days post hatch (dph) than at 90 dph in Asian seabass [15] and a similar trend was found in rainbow trout [16]. It is still uncertain whether heritabilities for growth traits increase or decrease with age in large yellow croaker.

Due to separate rearing of fullsib families before tagging, any common environmental effects could be confounded with additive genetic effect in the statistical analysis, and heritability estimates may thus be inflated in this study. However, when the common environmental effect was fitted in the model, it was not estimable, and ASReml automatically set the effect to zero in order to keep the solutions within the parameter space. Unfortunately, the hierarchical design used in this study is not well suited for separation of genetic and environmental effects pertaining to the same fullsib family. Still, the results may also indicate that the effect was indeed not large, which could be due to proper standardisation of the fullsib environments during the pre-tagging period, since the juvenile fish were reared in the sea most of the time, which to a large extent implied sharing equal seawater quality. Although common environmental effects have been shown to be considerable in many aquaculture species, ranging from 5% to 20% [17,18], a recent study also showed that this effect may to a large degree be due to maternal effects [19], as it was found that the effect accounted for as much as ~10% of the overall variance, despite eliminating all common environmental effects by use of a marker based tagging system, i.e. no separate rearing of families were required. The maternal effects may thus very well be smaller in marine species, like large yellow croaker, having smaller eggs than freshwater species.

Heterosis and reciprocal effect are other factors that may inflate the heritability estimates, and these effects could occur in the present design as we were doing crosses between the Mindong and Daiqu strains. Both effects are important to determine the optimum utilisation and setup for the different strain crosses in an eventual cross-breeding program. In tilapia, significant heterosis and reciprocal effects of growth traits have been observed in diallel crosses, e.g. 0-12% in Bentsen et al. and 6-12% in Said and Mekkawy [20,21]. However, we were not able to estimate any heterosis or reciprocal effects in our design, as only cross-strain mating and no pure-strain mating were performed. As mentioned above, the purpose here was to form a new mixed base population, and no crossbreeding scheme is planned in the future. Handling and mating of yellow croakers is still exceptional difficult, and even a simplified design, as in the present study, is a challenging task.

Genetic and phenotypic correlations among the growth traits of the fish were found all positive and high, especially between BW and BL. In an earlier study, genetic correlations among the same three traits were even higher than the present study, all above 0.95 [8]. Similar high genetic correlations have also been reported in other fish species; for instance in Nile tilapia (*Oreochromis niloticus*), the genetic correlation between body weight and body length at harvest was close to unity [22] , and in six months old coho salmon (*Oncorhynchus kisutch*) it was 0.98 ± 0.06 [23]. This may indicate that BW and BL are largely controlled by pleiotropic genes and selection could thus be conducted on either of the two traits with little difference in selection response. Considering easier measurement and higher heritability and CV for body weight, it is suggested that body weight should be used as the selection criterion.

The present genetic parameter estimates for large yellow croaker were based on records of six-month-old fish at tagging. However, growth until harvest is a more economically important trait for any breeding program. The validity of these genetic parameter estimates for growth at harvest time is uncertain, but in other cultured fish species, high positive genetic correlations between growth intervals have been reported. For example, the average genetic correlation for weight at various ages was 0.7 in European sea bass and 0.93 in red drum [24,25], although a study in rainbow trout showed that for distant life periods it can be as low as 0.24 [26]. The genetic parameters estimated for juvenile fish in this study could still be

useful predictors if pre-selection is performed instead of selection at harvest in yellow croaker, which will be the case if for instance a modern DNA-based tagging system is used. But the genetic correlation between the two growth stages is still needed and should be verified based on more experiments.

In the study, the highest coefficient of variation (CV) was found for BW, which is consistent with a previous study for 20 months old fish [8]. The average tagging weight was 66 ± 22 g for BW, which was larger than usual for other fish species at tagging, using 11 mm PIT-tags. For instance, in rohu carp (*Labeo rohita*), fingerlings at 8-15 g were found to be suitable for tagging with similar PIT tags [27]. The main reason for delayed tagging in our study was that the experiment experienced typhoon season during summer and the cages used were not storm-resistant.

CONCLUSION

In conclusion, the present results show that there is a potential to perform family selection for BW, BL and BH at an early age of large yellow croaker, as medium to high heritabilities were found for these traits at six months of age, and among these three traits, BW should be used as the selection criterion, since both the heritability and CV is highest for this trait. Future work and resources are needed to establish a full operational and secured breeding program for this fish species.

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Construction of Genetic Linkage Maps from a Hybrid Family of Large Yellow Croaker (*Larimichthys crocea*)

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Paper III

Construction of Genetic Linkage Maps from a Hybrid Family of Large Yellow Croaker (*Larimichthys crocea*)

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12 **ABSTRACT**

13 Consensus and sex-specific genetic linkage maps for large yellow croaker (*Larimichthys crocea*)
14 were constructed using samples from an F_1 family produced by crossing a Daiqu female and a
15 Mindong male. A total of 20,147 single nucleotide polymorphisms (SNPs) by restriction site
16 associated DNA sequencing were assigned to 24 linkage groups (LGs). The total length of the
17 consensus map was 1757.4 centimorgan (cM) with an average marker interval of 0.09 cM. The
18 total length of female and male linkage map was 1533.1 cM and 1279.2 cM, respectively. The
19 average female-to-male map length ratio was 1.2 ± 0.23. Collapsed markers in the genetic maps
20 were re-ordered according to their relative positions in the *ASM435267v1* genome assembly to
21 produce integrated genetic linkage maps with 9885 SNPs distributed across the 24 LGs. The
22 recombination pattern of most LGs showed sigmoidal patterns of recombination, with higher
23 recombination in the middle and suppressed recombination at both ends, which corresponds with
24 the presence of sub-telocentric and acrocentric chromosomes in the species. The average
25 recombination rate in the integrated female and male maps was respectively 3.55 cM/Mb and 3.05
26 cM/Mb. In most LGs, higher recombination rates were found in the integrated female map,
27 compared to the male map, except in LG12, LG16, LG21, LG22, and LG24. Recombination rate
28 profiles within each LG differed between the male and the female, with distinct regions indicating
29 potential recombination hotspots. Separate quantitative trait loci (QTL) and association analyses
30 for growth related traits in 6-month fish were performed, however, no significant QTL was detected.
31 The study indicates that there may be genetic differences between the two strains, which may have
32 implications for the application of DNA-information in the further breeding schemes.

33 **Keywords: large yellow croaker, RAD sequencing, linkage map, collinearity, recombination rate**

34

35 # INTRODUCTION

36 Large yellow croaker (*Larimichthys crocea*) has become an important aquaculture species in
37 southeast China, where Mindong and Daiqu are the two major strains farmed. Artificial breeding
38 of the Mindong strain started in 1985, while the Daiqu strain has been bred since 1999 (Chen et al.,
39 2018). In 2019, the total production of large yellow croaker exceeded 220,000 tons and accounted
40 for more than 12% of the cultured marine fish production of China (Yu et al., 2020a).

41 The production is supported by several breeding programs, of which the majority are based on
42 classical basic selection methods, like phenotypic selection (Chen et al., 2018). However, modern
43 breeding approaches, such as marker assisted selection (MAS), can further enhance the genetic
44 gain for economically important traits. MAS can greatly increase the efficiency if a sufficiently
45 large QTL is detected, typically through QTL linkage mapping and association studies (Zenger et
46 al., 2019). An outstanding example of MAS applied in aquaculture was the discovery of a QTL
47 imparting resistance to infectious pancreatic necrosis (IPN) in Atlantic salmon, accounting for
48 about 80% of the total variation in this trait (Moen et al., 2009; Houston et al., 2012). Information
49 from the QTL was used in selective breeding to generate IPN resistant fish, which now dominate
50 production in Norway, leading to a remarkable reduction in IPN outbreaks (Norris, 2017).
51 Subsequent studies have provided functional genomics data indicating that mutations in the
52 epithelial cadherin gene (*cdh1*) affect virion internalization (Moen et al., 2015), demonstrating the
53 power of genomic tools to help reveal the mechanistic basis for important traits. Although MAS
54 can be useful for some traits where major QTLs have been identified, most traits of economic
55 importance in aquaculture species (i.e., production traits) are assumed to be polygenic, and often
56 have low-to-moderate heritabilities (Zenger et al., 2019). As a result, application of MAS to

57 improve these complex traits may be inefficient. For such polygenic traits, *genomic selection* is a
58 viable alternative, based on genomic breeding values predicted on a genome-wide scale, allowing
59 even small QTLs to contribute (Meuwissen et al., 2001). For large yellow croaker, the estimates of
60 heritability for body weight (0.31 ± 0.06), body length (0.33 ± 0.06) and body height (0.41 ± 0.07)
61 in 6-month fish, and the genetic correlations between them ranged from 0.74 to 0.95 (Yu et al.,
62 2020b).

63 High-throughput sequencing has transformed genetics by making it relatively easy to generate
64 genome-wide genetic marker datasets, which are a prerequisite for QTL identification in MAS.
65 Significant progress was made through the discovery of cost-effective restriction-site associated
66 DNA sequencing (RADseq) based strategies (Baird et al., 2008). RADseq can generate medium
67 density SNP resources and has been successfully used in various fish species for genetic linkage
68 maps, QTL analysis and population genetics (Davey and Blaxter, 2010), e.g., in Atlantic salmon
69 (Houston et al., 2012; Gonen et al., 2014), channel catfish (Li et al., 2014) and Nile tilapia
70 (Palaiokostas et al., 2013).

71 Several genetic linkage maps for large yellow croaker have been developed using different
72 approaches (Supplementary **Table S1**). The first two genetic linkage maps made publicly available
73 were constructed using amplified fragment length polymorphism (AFLP; Ning et al., 2007) and
74 simple sequence repeats (SSR; Ye et al., 2014). However, next-generation sequencing technologies
75 have made detection of large numbers of genome-wide SNP markers relatively easy, and Ao et al.
76 (2015) constructed a SNP genetic linkage map with a total length of 5451.3 cM using RADseq,
77 while Xiao et al. (2015) constructed a genetic map of 2632 cM using RNA sequencing (RNAseq)
78 of expressed genes. More recently, Kong et al. (2019) constructed a double-digest restriction-site

79 associated DNA (ddRAD) based genetic map using 5261 SNPs with a total length of 1885.67 cM.
80 Despite using different approaches, these SNP linkage maps have one thing in common, as they
81 were all developed using only the Mindong strain.

82 Daiqu strain of large yellow croaker has been successfully cultured since 1999 and the
83 aquaculture production is on an industrial scale (Chen et al., 2018). Most consumers prefer lean
84 large yellow croaker, and the body shape has become an important economic trait (Dong et al.,
85 2019). The Daiqu strain has better performance for this trait, as the ratio of body length and body
86 height is significantly higher than for the Mindong strain (Huang et al., 2006). The Daiqu strain
87 also has later sexual maturation and better tolerance to lower temperatures than the Mindong strain
88 (Liu and Mitcheson, 2008; Miao et al., 2014). The offspring from a crossing between Mindong and
89 Daiqu displayed significant heterosis in body shape and growth of fish after 526 days (Li et al.,
90 2010). Our study therefore sought to develop a genetic linkage map in a crossed (F_1) family arising
91 from these strains.

92 The aim of this study was to construct consensus and sex-specific linkage maps based on a
93 hybrid family from the Daiqu and Mindong strains using RADseq, to compare the linkage map to
94 the latest physical map (*ASM435267v1*), and to perform a QTL analysis and association analysis
95 for growth-related traits.

96 MATERIALS AND METHODS

97 Mapping Family

98 A female (F_3 of wild Daiqu strain, from an aquaculture farm in Xiangshan, Zhejiang province) and
99 a male (approx. F_{10} of wild Mindong strain, from an aquaculture farm in Fuding, Fujian province)

large yellow croaker were crossed to generate a fullsib family (Yu et al., 2017a). One-hundred and twenty offspring were randomly selected at six months, and the following growth traits were recorded: body weight (BW), body length (BL) and body height (BH). Fin clips were preserved in 99% ethanol and sent to BGI Genomics Company (Shenzhen, China) for sequencing.

RAD Sequencing and SNP Calling

Library preparation was performed by BGI according to Baird (2008). In brief, individual genomic DNA samples were digested using the restriction enzyme *Pst I*, and the resulting fragments were ligated to a double-stranded Illumina sequencing primer containing a sample-specific barcode sequence. Libraries were then pooled and sheared by sonication, and fragments from 300 - 500 bp were separated by agarose gel electrophoresis and purified before ligating a Y-adapter to the sheared ends. Fragments including both barcode and Y-adapters were amplified with PCR to generate the final RAD libraries, which were then sequenced using a *Hiseq2000* platform to produce paired-end reads.

Raw reads were processed by BGI using the *Reseqtools* software package (https://github.com/BGI-shenzhen/Reseqtools) to remove adapter sequences and low-quality reads, and to de-multiplex the pool. The retained reads were analysed and genotyped using *Stacks* (Catchen et al., 2013) and in-house analysis pipelines, and RAD-tags with too low (< 2) or too high (> 100) sequencing coverage were excluded.

SNP Filtering and Linkage Map Construction

SNPs missing in > 10% of samples and minor allele frequency (MAF) < 0.05 were excluded using the PLINK software (Purcell et al., 2007). Markers were individually tested against the expected

121 segregation ratio, based on parental genotypes, and those showing significant segregation distortion
122 ($P < 0.05$, χ^2 test) were removed by PLINK.

123 The remaining SNPs were used to generate consensus and sex-specific maps using Lep-MAP2
124 software (Rastas, 2013). All SNP markers that passed filtering (n = 20,186) were used to produce
125 the consensus map, while those markers polymorphic in the father (n = 11,684) or mother (n =
126 11,838) were used to construct their respective sex-specific linkage maps. LGs were developed
127 using the *separate chromosomes* module, with a logarithm of odds (LOD) score ranging from 1 to
128 20. A LOD score of 9, which gave 24 LGs and the lowest number of single markers, was finally
129 selected. The option *sizeLimit =100* was used to generate linkage groups of size ≥ 100 markers.
130 The module *JoinSingles* could not assign any of the singular markers to any of the 24 LGs.
131 Eventually, 20,147 SNPs were ordered using the *OrderMarkers* module, which assign the markers
132 with paternal or maternal positions for the sex-specific maps. The option *sexAverage = 1* was
133 applied during execution of *OrderMarkers* to get positions for the consensus map. To avoid the
134 map distances being too long, especially when the number of markers per chromosome was much
135 higher than the number of individuals, the parameter *minError = 0.15* was used. Finally, the
136 *Kosambi* mapping function was used to calculate genetic distance between markers. The LG were
137 numbered by the SNP size of each LG (i.e., the LG with the largest SNP number was labelled LG1).
138 Illustrations of the consensus and sex-specific linkage maps were drawn using MapChart 2.32
139 (Voorrips, 2002).

140 **QTL Analysis and Association Analysis**

141 QTL analysis was initially performed using the QTL *IciMapping* software by the option *inclusive*
142 *composite interval mapping with an additive effect* (ICIM-ADD) (Meng et al., 2015; Li et al.,

143 The LOD threshold for QTL significance of each trait was determined by a permutation test (1000
144 replications) with a genome-wide significance level of 0.05. The permutation threshold method for
145 QTL mapping estimates the null distribution of the genome-wide maximum LOD score by
146 shuffling the phenotypes relative to the genotype data, breaking the association between the
147 phenotype and the genotypes (Churchill and Doerge, 1994). The genome-wide LOD thresholds are
148 calculated based on the 1-α quantiles of the genome-wide maximum LOD scores obtained from
149 the permutations, where α is the significance level (α=0.05 in our case).

150 As a complementary method for QTL mapping, a genome-wide association study (GWAS) was
151 performed using SNPs subjected to a more stringent quality filtering than that was applied for
152 linkage mapping to ensure a high QTL identification accuracy. Using PLINK, individuals
153 displaying more than 5% missing genotypes were removed. Also, SNPs were removed in cases
154 where missing genotypes > 5% across samples and Hardy-Weinberg P value (Fishers exact test) <
155 10^{-9}. The final SNP set used for GWAS thus included 16,570 SNPs from 74 individuals.

156 The genome-wide association analysis was performed using a mixed linear model equation on
157 BW, BL and BH by the *Genome-wide Complex Trait Analysis* (GCTA) program, with the *-mlma*
158 function (Yang et al., 2011). The following model was used:

159 $$\mathbf{y} = \mathrm{a} + \mathbf{b}\mathrm{x} + \mathbf{g} + \mathbf{e}$$

160 where **y** is the phenotypes (BW, BL, BH), a is the overall mean for each trait, **b** is the additive
161 genetic effect of the candidate SNP to be tested for association, x is the incidence matrix for the
162 candidate SNPs, **g** is the polygenic effect and **e** is the vector of random residual effects.

163 SNPs were considered genome wide significant when exceeding the Bonferroni threshold for
164 multiple testing ($\alpha = 0.05$) of $0.05/tg = 3.017502 \times 10^{-6}$, where tg = 16,570 (total number of
165 genome-wide SNPs); and SNPs were graded as chromosome-wide significant when Bonferroni
166 threshold for multiple testing ($\alpha = 0.05$) surpassed $0.05/tc = 7.246377 \times 10^{-5}$, where tc = 690
167 (average number of SNPs per chromosome). The genome-wide significant threshold used in this
168 study was $P \leq 3.017502 \times 10^{-6}$ ($-\log10(P) = 5.52$), while chromosome-wide significant threshold
169 was $P \leq 7.246377 \times 10^{-5}$ ($-\log10(P) = 4.14$). SNPs were visualised along the linkage groups using
170 the Manhattan function in the R package QQMAN (Turner, 2014).

171 **Collinearity Analysis: Genetic vs. Physical Map**

172 To explore the level of agreement between our consensus genetic map and a recently published
173 physical map, the large yellow croaker assembly, *ASM435267v1* (GenBank ID
174 GCA_004352675.1), the RAD-tag sequences (82 bp) from the consensus linkage map were aligned
175 to *ASM435267v1* using BLASTN (https://blast.ncbi.nlm.nih.gov/Blast.cgi) with the following
176 parameters: *expect value* $e \leq 1 \times 10^{-15}$, *identity* $\geq 95\%$, *matched length* ≥ 81 *bp, mismatches* ≤ 1
177 and *gap open* $= 0$. If a query sequence hit two or more loci in the physical assembly and the
178 difference between the 1st and 2nd smallest e-values was greater than 10^3, the 1st smallest e-value
179 was chosen to define the hit. Finally, 9885 SNPs from the consensus map hit the physical map.

180 The relative positioning of RAD sequences in the genetic map and the physical map were
181 graphically presented using *shinyCircos* (Yu et al., 2017b). The marker positions on genetic map
182 were multiplied by 4×10^5 for better visualisation of the Circos plot.

183

184 ## Adjusting Genetic Maps Based on the Physical Map

185 The collinearity analysis highlighted 107 SNPs whose assignment to LGs disagreed with their
186 physical assignment to chromosomes. Examples of this were seen in all LGs and, when detected,
187 SNPs were reordered according to the physical map. The adjusted SNP order in each linkage group
188 was used as an input of *evaluateOrder* option in the *OrderMarkers* module of Lep-MAP2, and the
189 genetic distances were recalculated using the *Kosambi* mapping function. The integrated consensus
190 and sex-specific linkage maps were drawn using *MapChart 2.32* (Voorrips, 2002).

191 Scatter plots were generated between the integrated linkage maps in cM distances and the
192 physical map in Mb distances by using the *ggplot2* package in R. Recombination rates throughout
193 the genome in the integrated female and male genetic maps were estimated using *MareyMap* online
194 (Siberchicot et al., 2017) with a computed sliding window size of 3.37 Mb. The threshold markers
195 number in a window was set to 8, the default value. The recombination rate changes throughout
196 the genome in the integrated female and male maps were visualised by using the *ggplot2* package
197 in R.

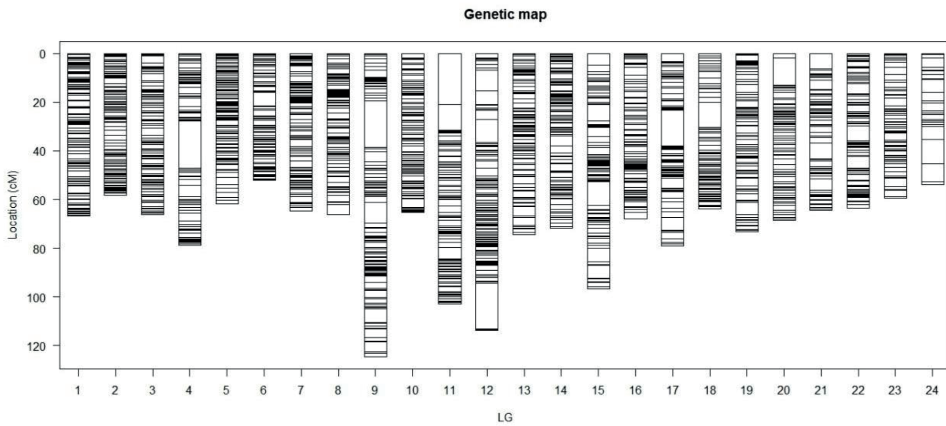
198 # RESULTS

199 ## Sequencing and SNP Filtering

200 Approximately 1.9 billion reads were produced after sequencing two parents and 120 offspring,
201 with each individual contributing roughly 15 ± 2.9 million reads. After reads filtering and RAD-
202 tag SNP detection, approximately 370,000 variants were detected within each individual. The
203 average heterozygosity rate was 32.5%. After filtering for segregation errors, MAF and missing
204 genotypes, a final set of 20,186 SNP markers was used for linkage map construction.

205 ## Linkage Map Construction

206 The SNPs were assigned to 24 LGs, in accordance with the haploid chromosome number (Lou et
207 al., 2015). The consensus map (**Figure1, Table1**) covered 1757.4 cM, with individual linkage
208 group lengths ranging from 51.9 cM (LG6) to 124.6 cM (LG9). The number of markers per linkage
209 group varied from 243 to 1230, with an average genetic distance between markers of 0.09 cM and
210 a standard deviation of 0.037 (**Table1**).



211 **FIGURE 1** The consensus linkage map for large yellow croaker. The dark bands show the density of the SNPs in the region of the LGs, whereas white bands show the regions with no SNPs.

212

TABLE 1 Key figures for the genetic linkage maps of large yellow croaker

	Consensus			Female		Male	
LG	No. of markers	Size (cM)	Average distance(cM)	No. of markers	Size (cM)	No. of markers	Size (cM)
1	1230	66.8	0.05	613	78.2	745	51.8
2	1125	58.3	0.05	783	65.7	716	43.1
3	1029	66.0	0.06	660	86.7	574	55.5
4	1000	78.7	0.08	654	66.8	503	83.5
5	997	61.8	0.06	626	72.6	629	49.4
6	973	51.9	0.05	543	54.2	548	43.8
7	967	64.6	0.07	532	67.2	558	50.1
8	949	66.3	0.07	458	70.2	647	60.4
9	899	124.6	0.14	609	68.3	375	58.0
10	875	65.2	0.07	484	68.6	505	49.4
11	850	102.8	0.12	475	64.4	468	51.8
12	837	113.7	0.14	533	68.6	410	59.3
13	831	74.4	0.09	480	58.5	480	52.8
14	817	71.9	0.09	589	71.8	377	50.7
15	814	96.6	0.12	297	70.4	589	52.9
16	791	68.0	0.09	387	61.2	526	55.2
17	789	79.0	0.10	417	70.4	476	45.6
18	789	63.9	0.08	616	55.2	566	48.6
19	787	73.2	0.09	589	54.2	283	56.6
20	741	68.4	0.09	539	54.2	358	46.1
21	693	64.5	0.09	378	50.5	392	55.3
22	689	63.6	0.09	237	39.8	528	58.0
23	432	59.3	0.14	225	65.7	273	50.5
24	243	53.9	0.22	114	49.8	158	50.8
Total	20147	1757.4	0.09	11838	1533.1	11684	1279.2

213 Separate male and female maps were constructed using segregating (heterozygous) markers
214 from each parent. The total length of the male linkage map was 1279 cM, and the total length of
215 the female map was 1533 cM (**Table1,** Supplementary **Figure S1**). In the female map, LG length
216 ranged from 39.8 cM (LG22) to 86.7 cM (LG3), and the SNP number per LG varied from 114 to

217 783. In the male map, the length of each LG varied from 43.1 cM (LG2) to 83.5 cM (LG4), and
218 the SNP number in each LG varied from 158 to 745. The average distance between markers for
219 female and male is thus 0.11 cM and 0.13 cM, respectively. The female-to-male length ratio ranged
220 from 0.7 (LG22) to 1.6 (LG3), with an average of 1.2 ± 0.23; most LGs in the female map were
221 larger than those in the male map, with the exceptions of LG4, LG19, LG21 and LG24.

222 QTL Analysis and Association Analysis for Growth Traits

223 The growth traits, BW, BL and BH, recorded in 120 offspring at six months of age, are presented
224 in **Table 2**. In the QTL analysis, the LOD threshold used was 8.81 for BW, 7.35 for BL, and 18.71
225 for BH. However, no QTL was above the LOD threshold for any of the growth traits, BW, BL or
226 BH (Supplementary **Figure S3**). In the GWAS analysis, the estimated genomic heritabilities for
227 the three traits were close to zero (**Table 3**). A total of 16,570 SNPs from 74 recorded individuals
228 were used, however, no SNPs crossed the genome or chromosome-wide significant level
229 (Supplementary **Figure S4**).

230

TABLE 2 Mean ± SD, range and coefficient of variation (CV) of growth traits at 6 months

231
232
233
234

	Body weight, g	**Body length, cm**	**Body height, cm**
Mean ± SD	45.2 ± 13.8	13.8 ± 1.6	3.6 ± 0.5
Range	20.8 – 89.3	9.5- 17.7	2.8 - 7.6
CV (%)	31	11	15

235

236 **TABLE 3** Estimates of variance components and heritability with standard errors (in parenthesis) using the genomic relationship matrix in GWAS by GCTA.

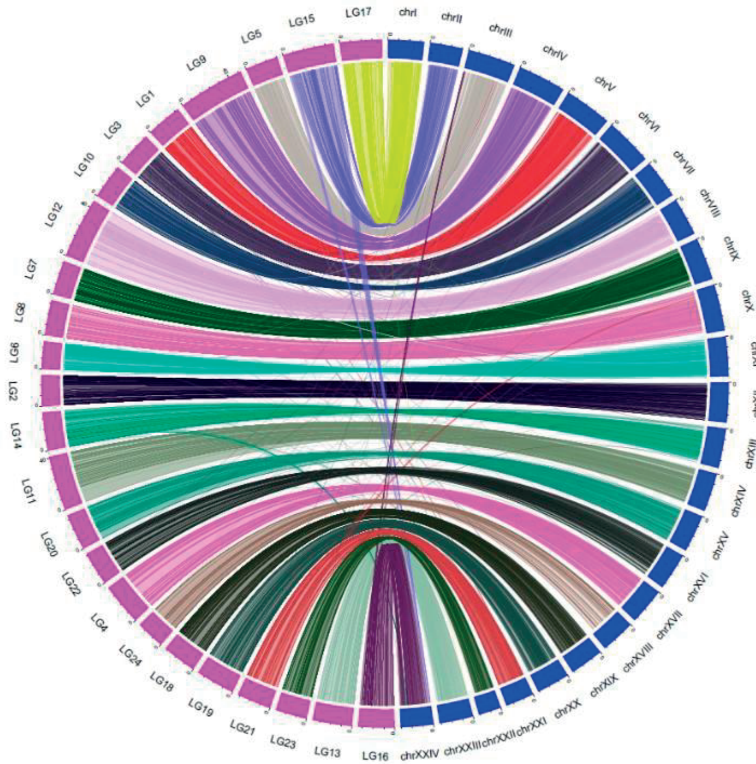
Traits	σ_g^2	σ_e^2	σ_P^2	*Genomic* h^2
BW	0.00022 (48.66)	194.5570 (42.94)	194.5572 (35.96)	0.000001 (0.25)
BL	0.000003 (0.69)	2.925069 (0.70)	2.925072 (0.52)	0.000001 (0.24)
BH	0 (0.08)	0.377716 (0.09)	0.37716 (0.07)	0.000001 (0.22)

237 **Note:** σ_g^2= Genetic variance; σ_P^2=Phenotypic variance; σ_e^2= Residual variance; h^2= Heritability.

238

239 ## Collinearity Analysis

240 In total, 9885 SNPs from the consensus map hit the physical map (*ASM435267v1*), but there were
241 107 SNPs hitting non-corresponding chromosomes. A collinearity analysis, comparing the
242 consensus linkage and physical maps, was performed (**Figure 2**). The average correlation
243 coefficient between the genetic map and the physical map was 0.78 ± 0.16 (Supplementary **Table**
244 **S2**). Each LG matches well with its corresponding chromosome of the physical map, with an
245 average matching percentage of 98.92% ± 1.5%. There were 7 LGs that showed no mismatch
246 between the genetic map and the physical map; LG7, LG9, LG10, LG17, LG18, LG20 and LG24
247 (Supplementary **Table S3**).



248

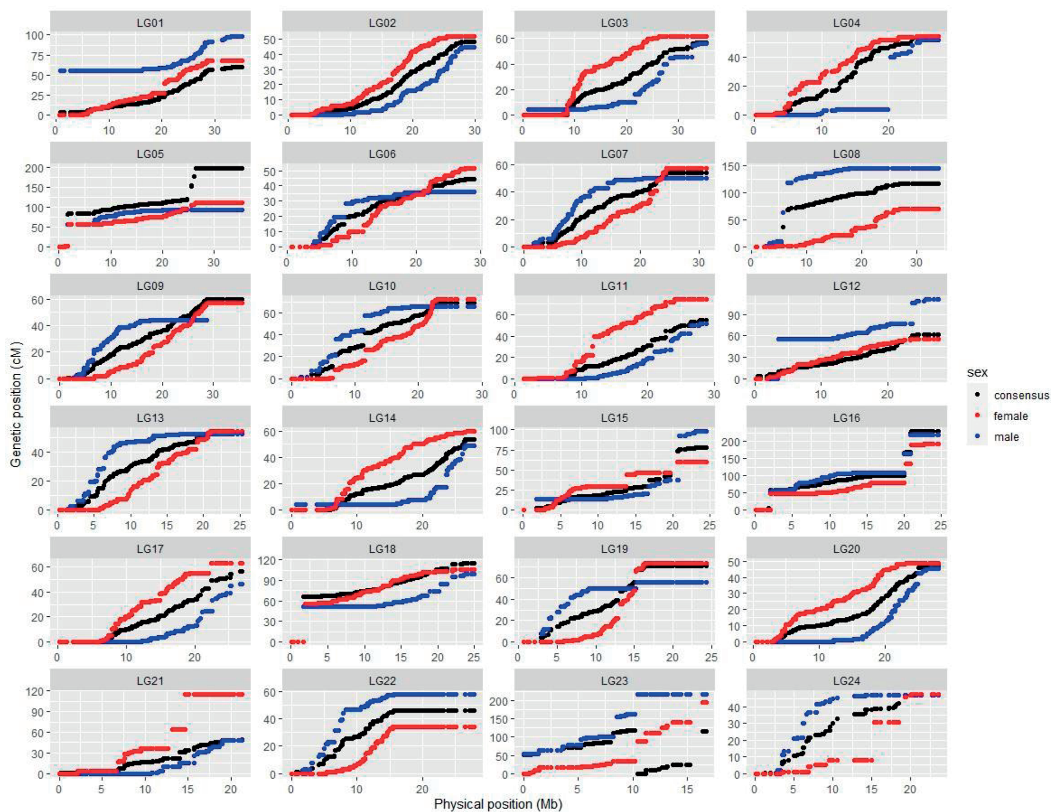
249 **FIGURE 2** Collinearity between the consensus genetic map (LG1-24) and the physical map (chr I-XXIV) of large yellow croaker.

250 ## Integration of Physical and Genetic Maps

251 SNP position information based on the *ASM435267v1* genome assembly was used to produce the
252 physically informed consensus, female and male linkage maps (Supplementary **Figure S2)**. A
253 summary of the integrated maps is shown in Supplementary **Table S3.** A comparison of map
254 positions between the integrated genetic and physical maps for different LGs is shown in **Figure**
255 **3**, in which most LGs exhibited sigmoidal patterns of recombination, with greater recombination
256 rates toward the middle and low recombination rates toward the ends of the chromosomes. Large

257 gaps or jumps can be seen in some of the plots, viewed from the x-axis or from the y-axis
258 (Supplementary **Table S4**). Viewed from the x-axis, representing the physical position, large gaps
259 were observed on LG24 (2.12Mb) and LG1 (1.9Mb), whereas viewed from the y-axis, representing
260 the genetic position, large gaps were observed e.g., in LG4 (36.36 cM) of the integrated male map
261 and in LG21 (50.37cM) of the integrated female map. The markedly large jump downward in LG23
262 of the integrated consensus map was due to the fragmented linkage group LG23.1 assigned to LG23
263 in this case. Recombination rates of the three integrated maps are shown in Supplementary **Table**
264 **S3**, and the recombination rate variation comparison of integrated female and male maps is
265 visualised in **Figure 4**. The average recombination rate in the female was 3.55 cM/Mb whereas it
266 in the male was 3.05 cM/Mb. The pattern of the recombination rates was different between male
267 and female in some LGs, as there was a higher recombination rate for the male than for the female
268 in the beginning of some LGs (e.g., LG09), whereas in other LGs the pattern was just opposite
269 (e.g., LG20).

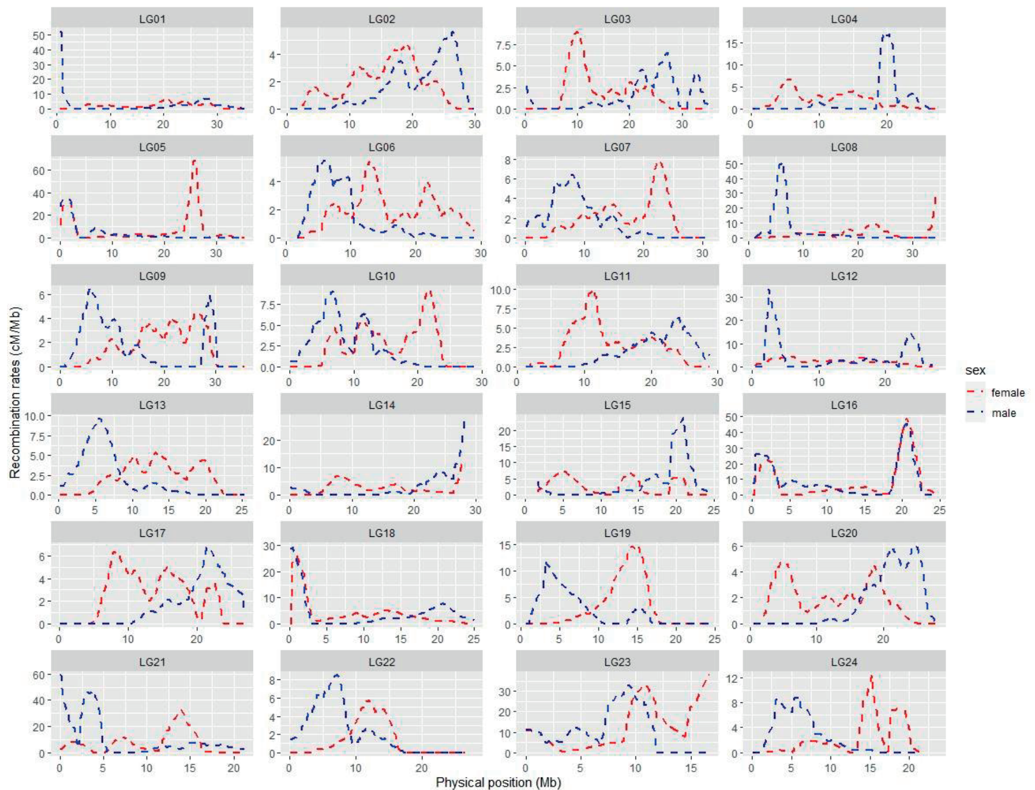
270



271

FIGURE 3 Scatter plots showing SNP linkage map positions (cM; y-axis) versus physical positions (Mb; x-axis) for integrated female (red), male (blue) and consensus (black) genetic maps. (Fragmented linkage group LG23.1 was assigned to LG 23.)

272



273 **FIGURE 4** Comparison of recombination rate variation throughout the genome in the integrated female (red) and male (blue) maps

274 # DISCUSSION

275 ## Linkage Map Construction and Collinearity Analysis

276 The total genetic length of the consensus linkage map in our study was 1757.4 cM. The genetic
277 map length (1885.67 cM) using the Mindong strain only, found by Kong et al. (2019), is slightly
278 larger than that of our study. However, the linkage map length, also using Mindong strain, found
279 by Ao et al. (2015) was 5451.3 cM, is much larger than in our study. The differences in total genetic

280 length could be caused by the mapping family used in our study, which was a cross between
281 Mindong strain and Daiqu strain. Suppressed recombination rates have also been reported in
282 rainbow-Yellowstone cutthroat trout (*Oncorhynchus clarkii bouvieri*) hybrids, there explained by
283 chromosome rearrangements (Ostberg et al., 2013). And in a hybrid cross of Human Pathogenic
284 Fungus, *Cryptococcus neoformans*, the linkage map length (197 cM) was much shorter than those
285 (1356.3 cM) observed in a single strain (Sun and Xu, 2007).

286 The average female-to-male map length ratio was 1.2 ± 0.23 in our study, indicating more
287 recombination events happening in females, which is consistent with an earlier study in large
288 yellow croaker by Ning et al. (2007). The phenomenon of heterochiasmy, i.e., sex differences in
289 recombination rates between the two sexes, has been found in many fish species. Higher
290 recombination rate in female fish, as in our case, was also reported in Atlantic salmon (1.38) (Lien
291 et al., 2011), gilthead sea bream (1.61) (Tsigenopoulos et al., 2014), Nile tilapia (1.2) (Joshi et al.,
292 2018) and Gasterosteus sticklebacks (1.64) (Sardell et al., 2018), where in all cases it seems that
293 the heterogametic sex has lower recombination rates. In our study, most LGs in the female map
294 were larger than those in the male map, whereas the male map was larger in LG4, LG19, LG21
295 and LG24. Similar cases were also found in other fish, such as the gilthead seabream and Nile
296 tilapia (Tsigenopoulos et al., 2014; Joshi et al., 2018). The molecular mechanisms for the sex
297 differences in recombination rates are still not well understood. The differences may be caused by
298 sexually antagonistic selection, meiotic drive in females, selection during the haploid phase of the
299 life cycle, selection against aneuploidy, or mechanistic constraints; however, no single hypothesis
300 can adequately explain the evolution of heterochiasmy in all cases (Sardell et al., 2020).

301 Sigmodal patterns of recombination in large yellow croaker, with greater recombination rates
302 toward the middle and lower recombination rates toward the ends, have also been seen in other
303 species, like Nile tilapia (Joshi et al., 2018), whereas, salmon, channel catfish, etc (Tsai et al., 2015;
304 Li et al., 2014) have shown opposite patterns, with higher recombination at the end of the LGs. The
305 segments with little or no recombination may suggest possible location of centromeres. The
306 karyotypes of large yellow croaker were earlier categorised into 10 pairs of sub-telocentric and 14
307 pairs of acrocentric chromosomes (Xu et al., 2017), implying that the centromeres are located at
308 the end of the chromosomes, matching the low recombination rates seen towards the end of these
309 LGs in our study. Recombination rate profiles within each LG also differed between males and
310 female, with distinct regions containing potential recombination hotspots.

311 Physical gaps, as viewed from the x-axis in **Figure 3**, indicate lack of SNPs in these regions.
312 One reason for these gaps could be massive repeat sites, unrecognisable by the *Pst I* enzyme during
313 RADseq. Identifying additional markers with a different enzyme should thus help to fill these gaps.
314 Another related reason could be the random and consequently partly uneven distribution of
315 detected markers across the genomes, which is a disadvantage of RAD based technologies. Thus,
316 RADseq usually generates medium density SNP linkage maps, leading to a low genome coverage
317 (Robledo et al., 2017). Furthermore, the strain used in the *ASM435267v1* genome assembly, called
318 DH2-L1, is a double haploid obtained by artificial gynogenesis from the Mindong strain only (Cai
319 et al., 2010), whereas the population used in our genetic map is a cross between Daiqu strain and
320 Mindong strain. The strain difference could thus be another reason for the physical gaps, as
321 chromosomal rearrangements, including deletions, duplications, inversions, and translocations,
322 could be different among strains.

323 Large jumps in some of the LGs were also viewed from the y-axis in **Figure 3**. These regions,
324 with significantly elevated recombination rates, may be due to recombination hotspots, insufficient
325 SNP coverage caused by the randomness of RAD sequencing explained above, and/or low level of
326 polymorphism in the F_1 family. A similar problem of large intervals was also presented in the
327 genetic linkage map of the small yellow croaker (*Larimichthys polyactis*), also from only one
328 fullsib family (Liu et al., 2020). Thus, use of multiple fullsib or halfsib families should be preferred,
329 as done for instance with the high-density linkage map developed in Nile tilapia using 41 fullsib
330 families (Joshi et al., 2018).

331 QTL Analysis and GWAS for Growth Traits

332 QTL analysis and GWAS are two types of strategies to detect potential causal genes for quantitative
333 traits. QTL analysis detects associations between marker intervals and phenotypes, while GWAS
334 identifies associations between single DNA markers and phenotypes, and thus the two methods
335 complement each other (Sonah et al., 2015).

336 For fish less than 10 months of age, the gonads are hard to assess only by naked eye observation
337 and there is hardly any gender difference to use for sex determination (Wang and Cai, 2018). Thus,
338 no gender information was available for the fish at 6 months in the present study. Growth
339 differences have already been observed between the Mindong and Daiqu strains and some
340 phenotype segregation may be expected in the F_1, but no significant QTLs or SNPs were detected
341 in our QTL or GWAS analysis. This was probably due to the complex genetic nature of the three
342 growth traits which generally have been found to be controlled by many genes, each with minor
343 effects. Also, the power of QTL analysis and GWAS will often not be sufficient with only one test
344 family, due to the categorical nature of QTLs, for which a significant variant may or may not be

345 present in any given family. For instance, the highly significant QTL variant that induced high
346 resistance to IPN virus in the study of Moen et al. (2009), was only present in ca 5 % of the breeding
347 nucleus. The QTL plot of BH is close to the threshold by 1,000 permutations, while the Manhattan
348 plot of BH is far from the suggestive threshold by Bonferroni correction, which has been reported
349 to be overly conservative in some cases (Kaler and Purcell, 2019). Also, no SNPs were identified
350 to be significantly associated with the BL/BD ratio or the BL/BH ratio in large yellow croaker
351 (Zhou et al., 2019; Dong et al., 2019). This may be due to low power in all these studies, but the
352 results correspond well with the assumed polygenic nature of these traits. However, Xiao et al.
353 (2015) identified several potential QTLs for growth traits (total weight, total length and total height)
354 by composite interval mapping using 72 individuals from one fullsib family. But LOD score
355 significance thresholds were not given in the plots in this study.

356 Using one F_1 fullsib family, as in the present study, Kong et al. (2019) identified seven
357 significant QTLs linked to white spot disease resistance. The probability of identifying the QTLs
358 in disease resistance traits could be higher than in growth traits, as it is often found that they are
359 controlled by some major QTLs (Fraslin et al., 2020). However, these studies, using one F_1 family,
360 only provide preliminary results of QTL mapping, and studies involving a more representative
361 sample of the breeding population are required to conduct a marker-assisted selection scheme. One
362 fullsib family is thus not ideal for identifying candidate genes, and a larger sample size and more
363 families should be used to improve the power and to reveal potential associations (Korte and
364 Farlow, 2013).

365

366 # CONCLUSION

367 A consensus genetic linkage map for large yellow croaker was constructed with 20,147 SNPs from
368 RAD sequencing, based on an F_1 family from Mindong strain and Daiqu strain. The total length of
369 the consensus map was 1757.4 cM with an average marker interval of 0.09 cM. The female-to-
370 male linkage map length ratio was 1.2. The map was adjusted based on the physical map, and
371 integrated consensus and sex-specific linkage maps were generated. The recombination pattern
372 mostly showed sigmoidal pattern of recombination. In most LGs, higher recombination rates were
373 found in the integrated female map, compared to the integrated male map. No significant QTLs for
374 growth related traits in fish at 6 months were found, probably due to the low detection power in
375 only one family and the polygenic and complex nature of growth traits that are controlled by many
376 genes with minor efforts. The present study indicates that there may be genetic differences between
377 the two strains Daiqu and Mindong, which may have implications for breeding programs using
378 DNA-information in a future selection scheme.

379 # SUPPLEMENTARY MATERIALS

380 **Figure S1**: Genetic maps of female (Daiqu strain) and male (Mindong strain); **Figure S2**: The
381 Integrated female, male and consensus genetic maps; **Figure S3**: QTL analysis for BW, BL and
382 BH; **Figure S4**: Manhattan plots for BW, BL and BH in GWAS.

383 **Table S1**: Published linkage maps of large yellow croaker; **Table S2**: Correlation between
384 consensus linkage map and the physical map; **Table S3**: A summary of the integrated maps; **Table**
385 **S4**: Max gaps of each LG in the integrated linkage maps.

386

387 ## ETHICS STATEMENT

388 The study and all experimental protocols were approved by the Animal Care and Use Committee
389 of Zhejiang Ocean University. The methods were carried out in accordance with local regulatory
390 welfare requirements.

391 ## ACKNOWLEDGEMENTS

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394

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557 SUPPLEMENTARY MATERIALS

558 Supplementary Figures

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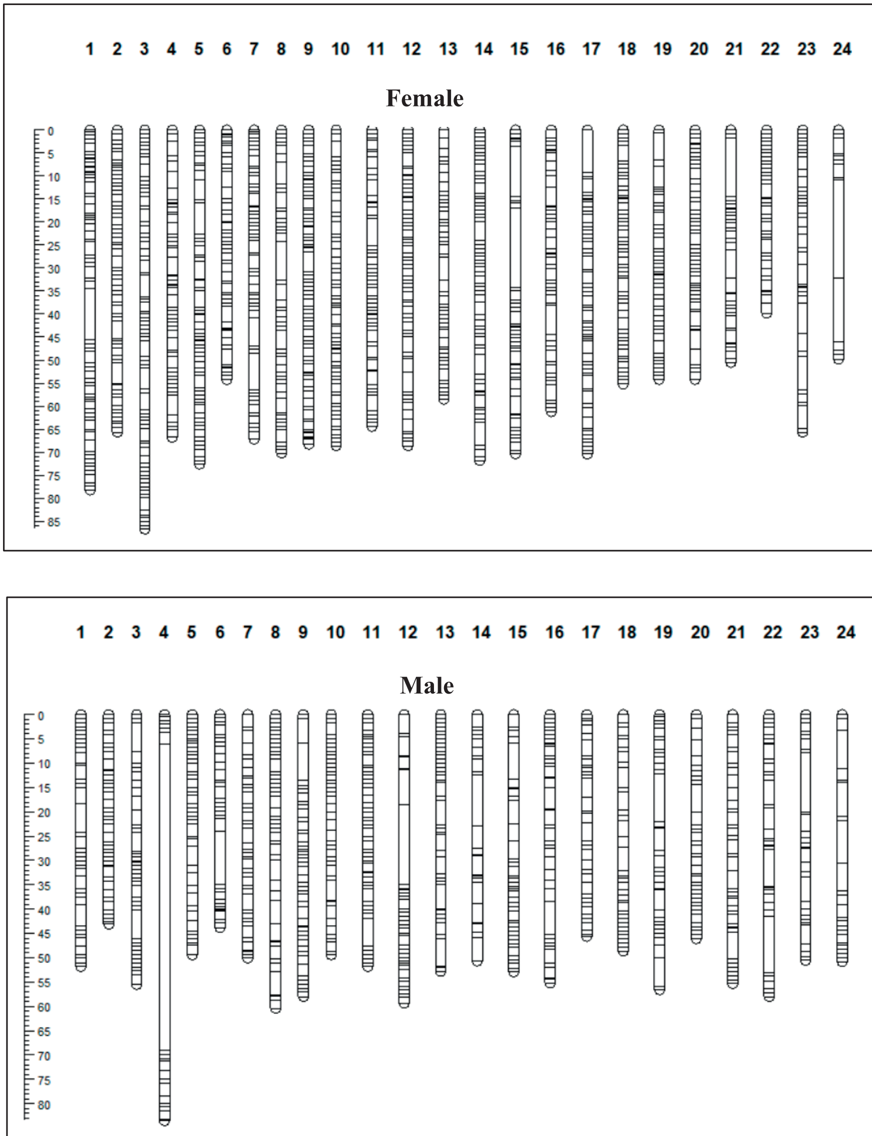

Figure S1 Genetic maps of female (Daiqu strain) and male (Mindong strain).

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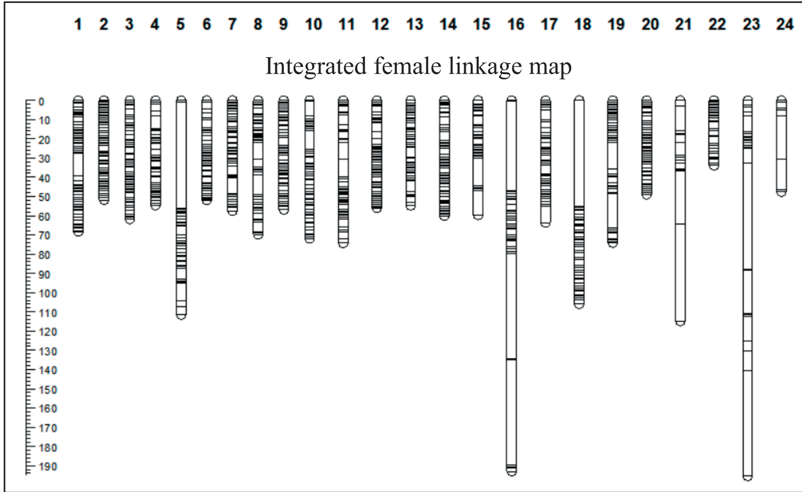

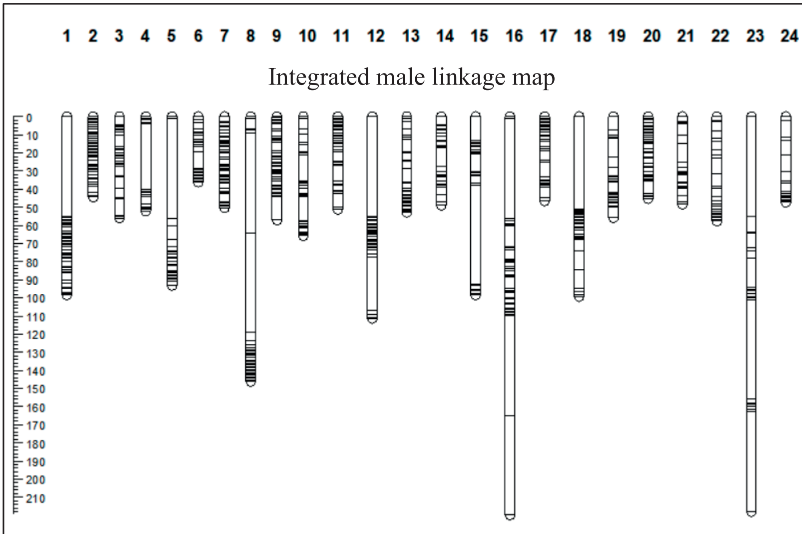

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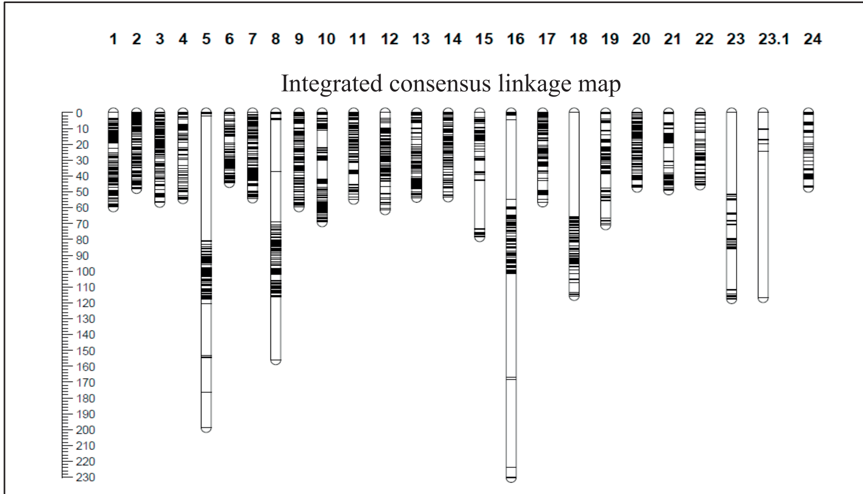

Figure S2. The Integrated female, male and consensus genetic maps

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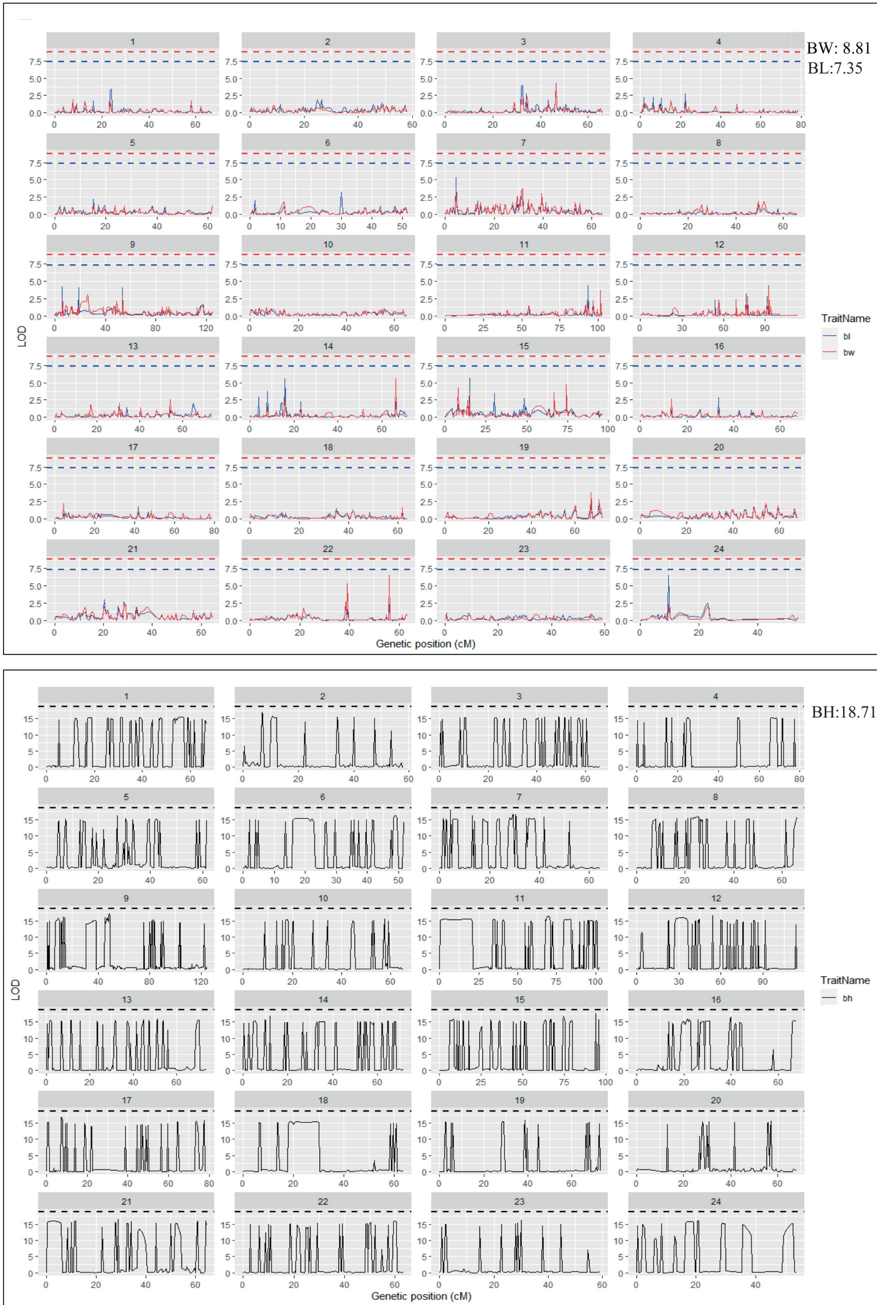

Figure S3. QTL analysis for BW (red), BL (blue) and BH (black). The y-axis shows LOD score and the x-axis shows the genetic position. The dashed lines represent the LOD threshold values (8.81 for BW, 7.35 for BL, 18.71 for BH)

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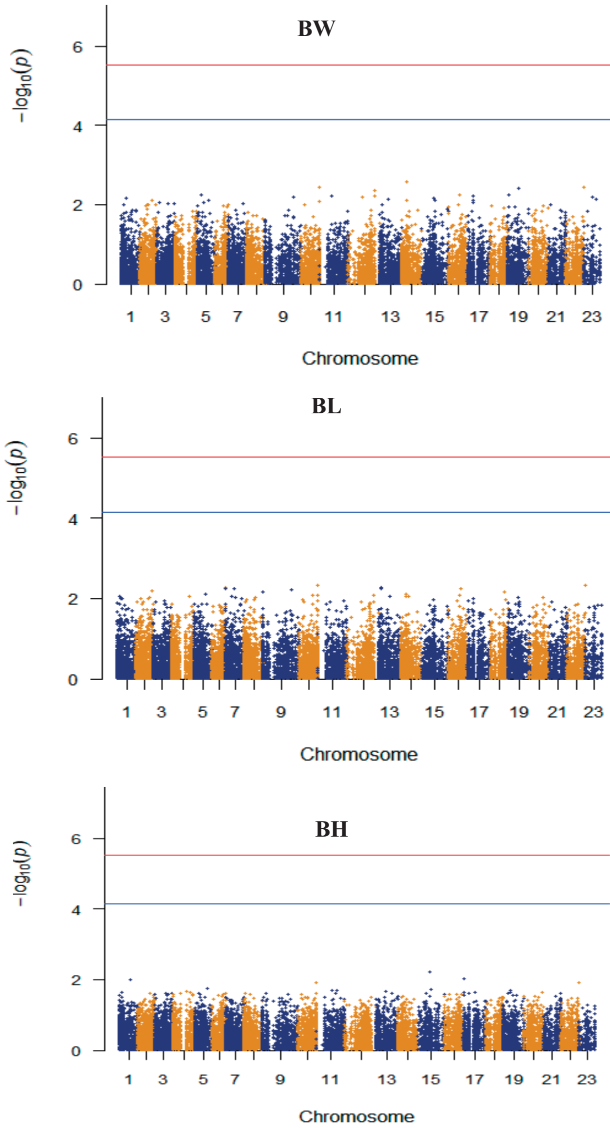

Figure S4. Manhattan plots for BW, BL and BH in association analysis.
The horizontal lines represent the genome-wide Bonferroni threshold (red) and chromosome-wide Bonferroni threshold (blue).

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Supplementary Tables

Table S1. Summary of published linkage maps for large yellow croaker

Author & Year	Type	Total length (cM)	Marker number and type	Average marker interval (cM)
Ning et al.,2007	female	2959.1	181 AFLP+7 SSR	15.7
	male	2205.7	153 AFLP+8 SSR	13.7
Ye et al., 2014	consensus	1430.8	289 SSR	5.4
Ao et al., 2015	consensus	5451.3	10,150 SNP	0.54
Xiao et al., 2015	consensus	2632	3448 SNP	0.76
Kong et al., 2019	consensus	1885.6	5261 SNP	0.36

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Table S2. Correlation between consensus linkage map and the physical map

LG	Chr	Correlation coefficient
1	V	0.87
2	XII	-0.86
3	VI	-0.91
4	XVII	0.88
5	III	-0.95
6	XI	0.94
7	IX	-0.92
8	X	-0.9
9	IV	-0.59
10	VII	0.93
11	XIV	-0.83
12	VIII	-0.58
13	XXIII	-0.86
14	XIII	0.87
15	II	0.52
16	XXIV	0.59
17	I	0.83
18	XIX	-0.7
19	XX	-0.91
20	XV	0.89
21	XXI	-0.79
22	XVI	0.62
23	XXII	0.3
24	XVIII	0.76
mean		0.78
sd		0.16

Note: Absolute values were used for calculation of mean and sd.

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Table S3 Integrated maps

LG	Chr	Total SNP hits	SNP matched with chr	SNP matched percentage (%)	Physical distance(Mb)	Integrated consensus map		Integrated female map		Integrated male map	
						Genetic distance(cM)	Recombination rates (cM/Mb)	Genetic distance(cM)	Recombination rates (cM/Mb)	Genetic distance(cM)	Recombination rates (cM/Mb)
LG1	V	677	670	98.97	34.285031	59.71	2.04	68.23	2.48	98.38	1.92
LG2	XII	547	545	99.63	29.262167	48.09	1.77	51.78	2.09	44.35	1.44
LG3	VI	497	493	99.20	35.068934	56.82	1.86	61.67	2.53	55.95	1.21
LG4	XVII	396	394	99.49	27.083572	54.51	2.04	54.64	2.24	52.07	1.65
LG5	III	492	491	99.80	33.805768	198.83	9.94	111.46	5.25	93.12	3.17
LG6	XI	488	487	99.80	28.328475	44.35	1.55	51.84	2.22	36.09	0.89
LG7	IX	545	545	100.00	31.102594	54.09	2.16	57.50	2.39	50.22	1.90
LG8	X	478	476	99.58	32.945456	116.19	3.11	69.85	3.23	146.06	2.61
LG9	IV	458	458	100.00	35.473149	59.67	1.87	56.90	1.76	56.97	1.76
LG10	VII	411	411	100.00	28.714631	69.11	2.68	71.88	3.34	65.74	2.04
LG11	XIV	422	415	98.34	28.613539	54.87	1.81	74.30	3.12	51.33	1.84
LG12	VIII	424	412	97.17	27.207652	61.50	2.10	55.88	2.42	111.33	3.07
LG13	XXIII	365	362	99.18	25.03279	53.68	2.20	54.67	2.68	52.84	1.75
LG14	XIII	428	412	96.26	27.78259	53.43	2.71	60.00	2.85	48.88	2.10
LG15	II	396	377	95.20	22.744536	78.50	2.71	59.73	2.92	98.42	2.31
LG16	XXIV	403	382	94.79	22.544341	230.30	9.89	192.98	8.14	219.75	8.15
LG17	I	424	424	100.00	26.701177	56.58	2.17	63.56	3.01	46.48	1.31
LG18	XIX	329	329	100.00	24.864925	115.57	3.60	105.96	3.57	99.24	2.76
LG19	XX	364	363	99.73	23.635925	70.98	3.48	74.13	4.31	55.77	2.00
LG20	XV	385	385	100.00	27.596533	47.27	1.89	48.97	1.98	45.18	1.78
LG21	XXI	309	304	98.38	21.206861	48.95	11.76	114.95	6.33	48.28	10.80
LG22	XVI	325	322	99.08	27.727283	45.75	1.87	33.87	1.84	57.55	1.91
LG23	XXII	206	205	99.51	15.23173	116.91	2.96	195.40	12.46	217.75	11.90
LG24	XVIII	116	116	100.00	23.404721	47.27	2.50	47.53	2.06	47.34	2.84
Total		**9885**	**9778**	–	–	**1842.93**	–	**1837.67**	–	**1899.10**	–
Mean		–	–	**98.92**	–	**76.79**	**3.36**	**76.57**	**3.55**	**79.13**	**3.05**
SD		–	–	**1.50**	–	**46.89**	**2.78**	**40.37**	**2.37**	**49.69**	**2.85**

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Table S4 Max gaps of each LG in the integrated linkage maps

LG	Max physical gap in each LG (Mb)	Max genetic gap in each LG(cM)		
		consensus	female	male
LG1	1.9	3.67	11.66	54.93
LG2	0.5	1.81	3.30	3.33
LG3	0.82	2.78	3.34	9.04
LG4	0.8	3.50	6.62	36.36
LG5	1.21	78.74	54.93	54.93
LG6	1.01	4.49	4.17	9.28
LG7	0.83	3.10	7.75	4.14
LG8	1.16	32.28	10.20	54.93
LG9	0.71	2.72	4.25	12.77
LG10	1.64	11.86	9.60	14.44
LG11	0.68	6.34	9.21	8.92
LG12	0.91	4.17	4.35	54.93
LG13	0.59	3.30	5.15	7.48
LG14	1.7	2.23	4.55	9.98
LG15	1.65	30.52	14.34	54.93
LG16	0.57	65.08	54.93	54.93
LG17	1.4	6.51	8.43	8.30
LG18	0.69	65.70	54.93	50.89
LG19	0.84	11.17	17.89	10.38
LG20	0.77	3.16	2.67	6.51
LG21	0.61	8.59	50.37	10.17
LG22	1.37	4.05	3.66	8.58
LG23	1.18	92.67	54.93	54.93
LG24	2.12	4.94	22.81	9.22
Average	**1.07**	**18.89**	**17.67**	**24.83**
sd	**0.45**	**26.87**	**19.25**	**21.67**