UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA

ANEXO II

UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA

Instituto Universitario de Sanidad Animal y Seguridad Alimentaria

PROGRAMA DE DOCTORADO TITULADO "ACUICULTURA: PRODUCCIÓN CONTROLADA DE ANIMALES ACUÁTICOS"

Tesis Doctoral presentada por **Dña. Davinia Negrín Báez**

Dirigida por **la Dra. María Jesús Zamorano Serrano**

y la Dra. Ana Navarro y Guerra del Río

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Las Palmas de Gran Canaria, 2014

Grupo de Investigación en Acuicultura

UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA

Instituto Universitario de Sanidad Animal y Seguridad Alimentaria

TESIS DOCTORAL

DETECCIÓN DE QTLs DE DEFORMIDADES ESQUELÉTICAS EN DORADA (*Sparus aurata* L.): HERRAMIENTAS MOLECULARES Y ANÁLISIS DE SEGREGACIÓN

Davinia Negrín Báez

Las Palmas de Gran Canaria, 2014

María Jesús Zamorano Serrano, Profesora Contratada Doctora de la Universidad de Las Palmas de Gran Canaria, perteneciente al Departamento de Patología Animal, Producción Animal, Bromatología y Ciencia y Tecnología de los Alimentos de la Universidad de Las Palmas de Gran Canaria,

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INFORMAN:

Que Davinia Negrín Báez, Licenciada en Veterinaria por la Universidad de Las Palmas de Gran Canaria, ha realizado bajo nuestra dirección y asesoramiento, el presente trabajo titulado "DETECCIÓN DE QTLS DE DEFORMIDADES ESQUELÉTICAS EN DORADA (*Sparus aurata* L): HERRAMIENTAS MOLECULARES Y ANÁLISIS DE SEGREGACIÓN, el cual consideramos reúne las condiciones y la calidad científica para optar al grado de Doctora.

En Las Palmas de Gran Canaria, a 18 de Septiembre de 2014.

Fdo.: Mª Jesús Zamorano Serrano **Fdo.: Ana Navarro y Guerra del Río**

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Dedicada a
mi madre

DOCTORAL THESIS

DETECTION OF QTLs FOR SKELETAL DEFORMITIES IN GILTHEAD SEABREAM (*Sparus aurata* L.): MOLECULAR TOOLS AND SEGREGATION ANALYSIS

Davinia Negrín Báez

Las Palmas de Gran Canaria, 2014

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Abstract

The aim of the present study was to analyze the existence of QTLs (*Quantitative Trait Loci*) affecting the most important skeletal deformities in gilthead seabream (*Sparus aurata* L.), which could be used as a tool to minimize the prevalence of these deformities in this species under a genetic breeding program. The incidence of these anomalies in this species supposes important economic losses in the industry, both hatchery and on-growing companies, so abnormal fish have to be removed at different points of the production line.

In a first step, a set of multiplex PCR reactions containing 138 microsatellite markers of gilthead seabream genetic map, which were redesigned to be amplified under the same PCR conditions, was developed. The final set was conformed by 13 new multiplex PCR reactions (named ReM*sa*1 to ReM*sa*13) containing 106 of these microsatellite markers. This covered 100% of the linkage groups, and, after being successfully validated, this set constitutes a powerful tool to search QTLs in this species.

In order to find a family structure to facilitate the detection of QTLs, the prevalence of important and severe skeletal deformities was analyzed in different populations of gilthead seabream in three large-scale experiments. These deformities were lack of operculum, lordosis, vertebral fusion and LSK complex (lordosis-scoliosis-kyphosis). In Experiment 1, fish were obtained from a mass spawning and, at 111 days, they were sorted in deformity terms. At 509 days post-hatching, 900 fish were analyzed: 846 fish on-grown in a farm and 54 LSK fish selected in the initial sorting process and reared separately in a tank. All the individuals with this deformity were in six of 89 families represented, which showed a significant association with the deformity. So, five of these six families were selected for QTL mapping. In Experiment 2, fish were obtained from a mass spawning but no fingerling sorting process was carried out. Although at small size, the prevalence of lack of operculum in the

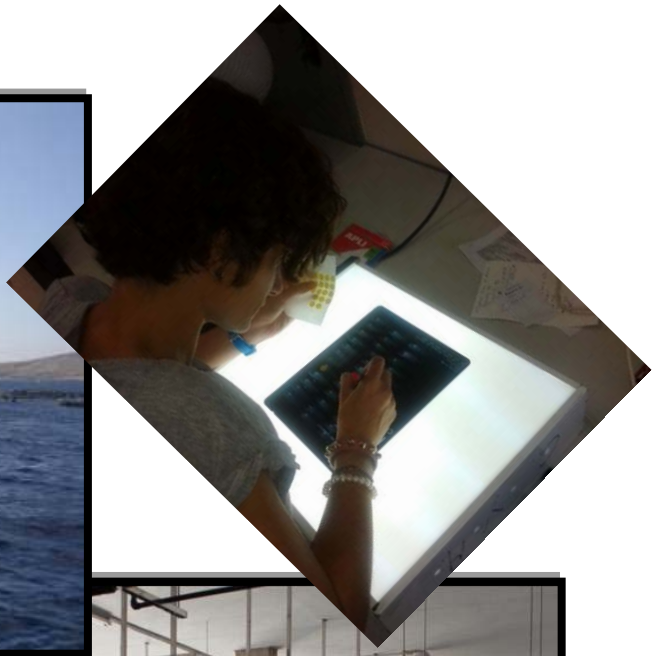
offspring was independent of family or breeder, at 539 days post-hatching, significant relationships between a sire, a dam, and two of the families, and prevalence of this deformity in the offspring were found. In fact, 48.94% of individuals showing this deformity were descendants of this sire. Therefore, all the descendants of this sire (six half-sib families) were selected for QTL mapping. In Experiment 3, designed matings were proposed: sires suffering from lordosis or operculum or vertebral fusion deformities were mated with non-deformed dams. A mass-spawning was considered as a control mating. At 129 days post-hatching, a total of 11503 offspring were visually analyzed for deformities. A significant relationship between the prevalence of each deformity and the mating of breeders suffering from the same deformity was observed. However, a significant prevalence of lack of operculum was also observed in mating with lordotic sire, thus, revealing an important influence of environmental factors on this deformity presence, while lordosis and vertebral fusion deformities are related to family structure.

Finally, a search for QTLs for five skeletal deformities in 12 gilthead seabream families was performed. This search was divided into three studies. All the fish were genotyped by using the set of multiplex PCRs ReM*sa*1-13. In Study 1, five families selected in Experiment 1 were analyzed for LSK complex deformity. 14 LSK-related significant QTLs were found and four of them, located in linkage groups (LG) 5, 8, 17 and 20, were the most solid with an extremely large effect. A strongly association between the genotype of closely located markers of these QTLs and the phenotype was observed. In Study 2, the paternal half-sib family selected in Experiment 2 was analyzed for lack of operculum deformity. Two of the four QTLs detected for this deformity were significant QTLs, and they were located in LG9 and LG10. Both QTLs showed a large effect, and a significant association between lack of operculum deformity and male allelic segregation was observed in one of them. In Study 3, a full-sib family from a mating of deformed breeders was analyzed for different deformities.

Three of the seven QTLs found were significant for vertebral fusion in LG21, for lordosis in LG9 and for jaw deformity in LG13, but they should be confirmed in other families.

The results of the present work confirm the genetic origin of these skeletal deformities in gilthead seabream, as well as their relationship with family structure. In addition, QTLs for these deformities were detected, as well as a significant association between allelic segregation of their closely located molecular markers and phenotype. This represents a major step towards the location of genes that determine the presence of skeletal deformities in this species. The above reveals the potential of applying molecular genetics-based knowledge from identificating specific QTLs, to reduce the prevalence of these deformities in gilthead seabream industry within a genetic breeding program.

1. GENERAL INTRODUCTION



1.1. Species of Study: Gilthead Seabream

1.1.1. Taxonomy and Nomenclature

Gilthead seabream is a marine species that belongs to *Sparidae* family, more concretely to genus *Sparus* (*Sparus aurata*, Linnaeus, 1758). It is taxonomically classified as follows:

Kingdom	*Animal*
Phylum	*Chordata*
Subphylum	*Gnathostomata*
Class	*Actinopterygii*
Subclass	*Teleostei*
Superorder	*Neognathi*
Order	*Perciformes*
Family	*Sparidae*
Genus	*Sparus*
Species	*Sparus aurata*

Their vernacular names in different languages are the following:

Spanish	*Dorada*
English	*Gilthead seabream*
French	*Dorade*
German	*Goldbrasse*
Italian	*Orata*

1.1.2. Distribution and Habitat

The geographical range of this species includes the eastern shores of the Atlantic Ocean, from Great Britain to Cape Verde, and the Mediterranean Sea. Being euryhaline and eurythermal, this species inhabits coastal areas of marine environments, coastal lagoons and estuarine areas, mainly during the early stages of their life cycle. Gilthead seabreams conduct a trophic migration, they spawn in the sea in winter, and juvenile fish migrate in early Spring

towards more protected areas, where an abundance of food is available and temperature is mild; and they are back to open sea in late autumn to reproduce (Moretti *et al*., 1999).

1.1.3. Anatomy

Gilthead seabream is a teleost fish whose body is ovally shaped, lightly compressed and compact. Its head shows a regularly-curved profile and small eyes. Its mouth is subterminal, lightly oblique and shows big lips. It shows from four to six front canine-like teeth in each mandible, followed by blunt teeth that transforms progressively to molars distributed in 2-4 rows (the teeth in the two external rows are stronger than those of the other rows). Gilthead seabream shows a silver grey body, with a big black patch from the origin of the lateral line to the superior margin of the operculum. It shows a golden band between the eyes, the limits of which are marked by two darker areas (not well defined in young individuals); dark longitudinal lines on body sides; a dark band over the dorsal fin; as well as, black tail fin borders and fork (Fig. 1).

Figure 1. Side view of the external appearance of an adult gilthead seabream

1.1.4. Feeding and Reproduction

Gilthead seabream is a mainly carnivorous fish, which preys on benthic species (bivalves and gastropods), crustaceans and small fish. Generally, it is considered a fast grow species in nature, as it reaches 300 g in their second year of life and approaches to 600 g in

their third year of life. Gilthead seabreams can grow until a total length of 70 cm and a weight of 5 Kg (Castelló-Orvay, 1993).

Its reproduction is protandrous hermaphrodite, at first it grows as a male and when it is two or three years old it becomes a female. Its breeding season is a relatively extended period, more concretely, from October to December, when days and daylight hours are shorter in Western Mediterranean (Arias, 1980), and from November to February in Eastern Mediterranean Sea, its breeding season occurs later, from November to February (Ben-Tuvia, 1979). Their eggs are planktonic, spherical and transparent, with a diameter of about 1 mm and containing one or two drops of oil inside; and their production ratio is from 500,000 to 6,000,000 eggs per kilogram of laying female fish (Cejas *et al*., 1992).

To spawn during the winter, gilthead seabreams migrate to coastal areas, as they offer more protection, an abundance of food, and a milder temperature (Tropical Migration). Later, at the end of autumn, they migrate back to open sea to start their breeding season as adult fish.

1.2. Aquaculture Production of Gilthead Seabream

1.2.1. Aquaculture Production at European and Global Level

According to the data published by APROMAR (2014), there is gilthead seabream aquaculture production in 19 countries, being the main producers in 2013 the following: Greece (about 75,000 tons (t) (41.7% of the global production)), Turkey (41,700 t (23.2%)) and Spain (16,795 t (9.3%)). Gilthead seabream is also produced in Italy, Egypt, France, Cyprus, Portugal, Croatia, Malta and Tunisia, and there are also incipient productions in Albany, Dominican Republic, Morocco, United Arab Emirates, Bosnia, Oman, Libya and Kuwait. Progression of gilthead seabream aquaculture production in the Mediterranean area and in the rest of the World from 1983 to 2013 can be observed in Fig.2.

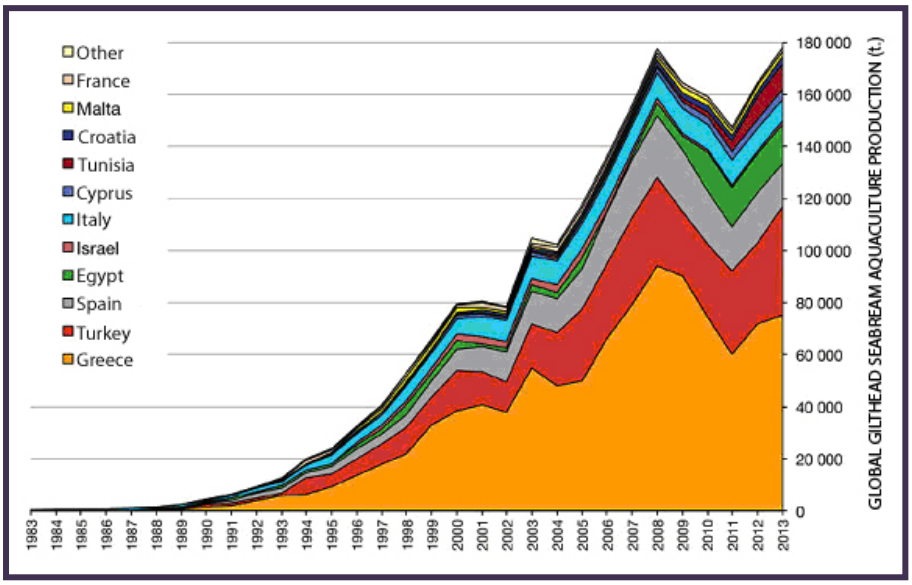


Figure 2. Progression of gilthead seabream aquaculture production in the Mediterranean area and in the rest of the World from 1983 to 2013 (APROMAR, 2014**)**

1.2.2. Aquaculture Production in Spain

The production of gilthead seabream is the highest fish production in Spain in 2013 (16,795 t), followed by the production of rainbow (*Oncorhynchus mykiss*) (16,732 t) and of European seabass (*Dicentrarchus labrax*) (14,707 t).

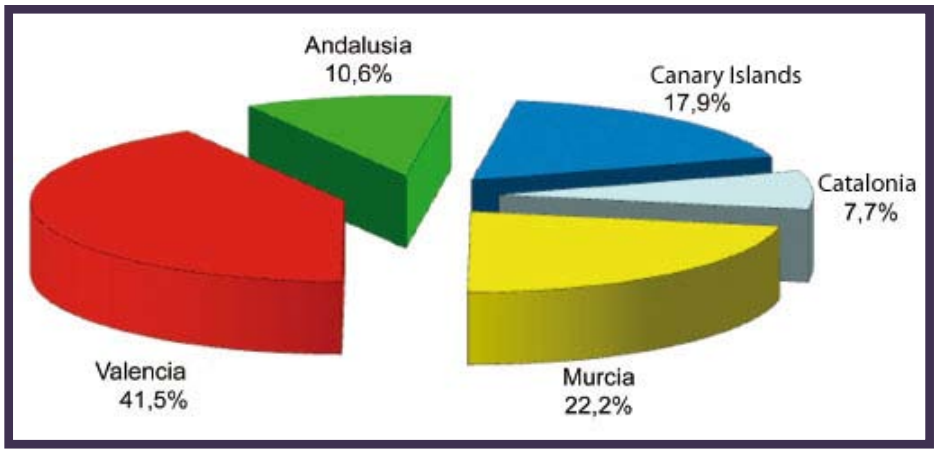


Figure 3. Distribution of the gilthead seabream productions in Spain for Autonomous regions in 2013 (%) (APROMAR, 2014)

In 2013, the aquaculture production of gilthead seabream in Spain was led by Autonomous region of Valencia (6,974 t (42% of the total production)), followed by Murcia (3,730 t (22%)), the Canary Islands (3,016 t (18%)), Andalusia (1,786 t (11%)) and Catalonia (1,292 t (8%)) (APROMAR, 2014). Production of gilthead seabream in Autonomous regions

of Spain is shown in Fig. 3; as well as, production in Canary islands from 1998 to 2012 is shown in Fig. 4.

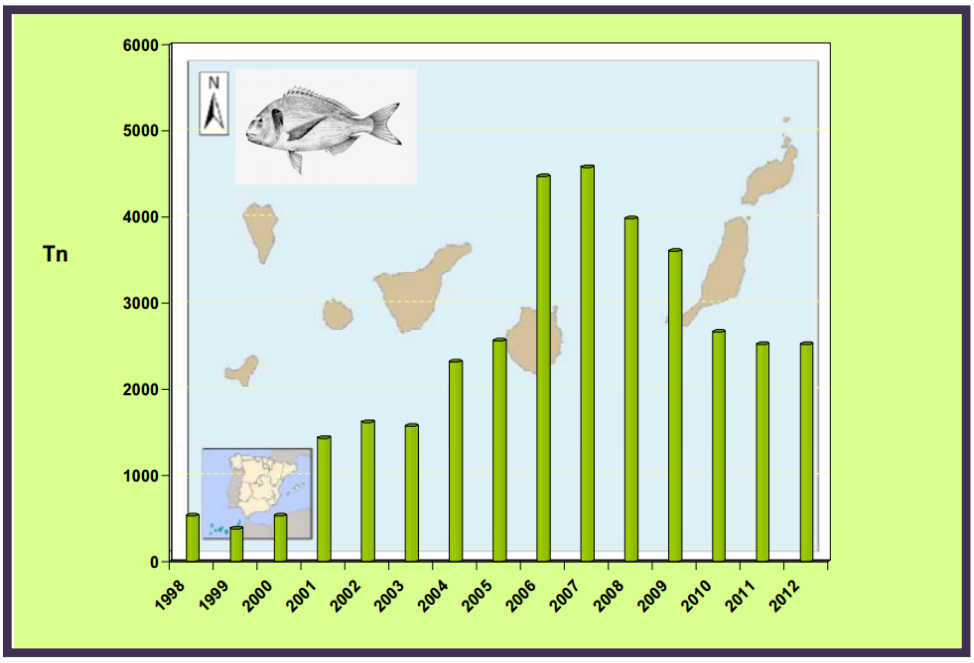


Figure 4. Aquaculture production of gilthead seabream in the Canary Islands. Information provided by Ministerio de Agricultura, Alimentación y Medio Ambiente, Secretaría General de Pesca, Junta Nacional Asesora de Cultivos Marinos (JACUMAR, 2014)

With respect to juvenile fish production in Spain, it reached 51.4 millions units in 2013, which is 6.5% lower than those produced in 2012. This production of juvenile fish in Spain concentrates on Comunidad Valenciana, the Balearic Islands, Cantabria and Andalusia. Nevertheless, to produce gilthead seabream at commercial size in Spain, it is necessary to import juvenile fish. The first sale value of commercial size gilthead seabream in Spain was 72,165,279 € in 2012 (JACUMAR, 2014).

1.3. Justification of the Study

At present, 95.3% of the total amount of gilthead seabream consumed in Spain comes from aquaculture. This notable development of gilthead seabream aquaculture production entailed an increase in their high market competence and, in consequence, a decrease of their final product sale price. So, companies had to minimize costs and to increase their production

in order to maintain their benefits. In this regard, genetic improvement has been recognized as an important factor to develop a profitable, efficient and sustainable aquaculture (Gjedrem, 2005; Rye *et al*., 2010).

Recently, the project PROGENSA® has developed at national scale a pilot program to improve gilthead seabream aquaculture production in collaboration with on-growing companies and research centers from four autonomous Spanish regions. It has achieved the articulation of the genetic tools and protocol that were needed to establish a genetic breeding program without interfering with the industry performance (Afonso *et al*., 2012). Within a genetic improvement program, the identification of Quantitative Trait Loci (QTL) is a way to improve the selection efficiency. QTL is a cromosomic region (one or more gens) that determines a quantitative trait. The identification of QTLs also allows the Markers Assisted Selection (MAS), which is based on the linkage disequilibrium of these molecular markers and the phenotype of a specific studied trait in the population (Pérez-Enciso and Toro, 2007). The advantages of MAS selection are notable as compared with the traditional selective breeding, so it is an increasingly important tool in genetic animal breeding (Yue, 2014).

To get an efficient QTL detection, it is necessary to have the following: a genetic linkage map with a high level of molecular markers saturation, proper phenotypic data and an adequate genetic statistic model (Lynch and Walsh, 1998). In gilthead seabream, a linkage map based on 204 microsatellite markers and 26 linkage groups is available (Franch *et al*., 2006). Microsatellite markers are probably the most useful molecular markers for medium-high density maps, as they are highly polymorphic and codominant, they are distributed throughout the genome and they can be easily analyzable by the Polymerase Chain Reaction (PCR) (Bouza *et al*., 2007).

Currently, MAS has not played a major role in most of genetic improvement programs in aquaculture industry. However, a wide number of studies have revealed the existence of QTLs for the most interesting traits in different species (reviewed by Yue, 2014). To search

and detect QTLs, it is necessary a high number of markers, in order to cover as many loci as possible, and a high number of genotyped animals. An efficient way to reduce reaction costs and to minimize genotyping errors is to use multiplex PCR (Navarro *et al*., 2008). In gilthead seabream, it has also been reported a number of QTLs that are related with commercially important traits, such as growth, sex determination, several morphometric traits, resistance to pasteurellosis and confinement stress; these studies were conducted by microsatellite genotyping (Massault *et al*., 2010; Boulton *et al*., 2011; Loukovitis *et al*., 2011, 2012 and 2013). Nevertheless, none of these QTLs has been determined by multiplex PCR, as there is not any gilthead-seabream-specific multiplex PCR considering a high number of microsatellite markers located into the genetic map and adequate to identify QTLs.

On the other hand, growth and deformities are the most economically important trait for the industrial production of gilthead seabream (Georgakopoulou *et al*., 2010). In gilthead seabream industry, in which products are mainly commercialized as whole fish, deformed fish affect negatively to the turnover of hatcheries and on-growing companies. In fact, this industry invests heavily in reducing the prevalence of deformities, and they discard deformed fish at different steps of the production cycle (Afonso and Roo, 2007).

In aquaculture, deformities altering the fish appearance are considered the most important, since they affect directly to their production traits (Gjerde *et al*., 2005; Kause *et al*., 2005; Afonso and Roo, 2007; Fjelldal *et al*., 2009). Among them, skeletal deformities, such as neurocranium or head, vertebral column and appendicular skeleton, are the most relevant deformities. Head deformities include those affecting jaw and operculum complex (lack of operculum). Scoliosis, kyphosis, lordosis and vertebral fusion are the most frequent vertebral column anomalies (reviewed by Boglione *et al*., 2013). A high number of studies has determined that physiological, environmental, xenobiotic and nutritional factors are linked to fish deformities appearance (reviewed by Bardon *et al*., 2009), but just a few of them contributed to their genetic determination. In gilthead seabream, Afonso *et al*. (2000) found a

significant family association for a triple column deformity (LSK complex, lordosis-scoliosis-kyphosis), Astorga *et al*. (2004) estimated the heritability of any type of deformity, and Lee-Montero *et al*. (2014) estimated the heritabilities of lack of operculum and of vertebral deformity. These studies suggest that skeleton deformities variability are influenced by the environment but could be also explained by a genetic origin. In this regard, a MAS selection would be an efficient tool to optimize the genetic improvement, being especially profitable for traits that are difficult to measure on offspring, that exhibit low heritability and/or that are expressed later in development (reviewed by Yue, 2014), such as skeletal deformities. However, there is no QTL described for any type of deformity in gilthead seabream.

1.4. Objectives of the Study

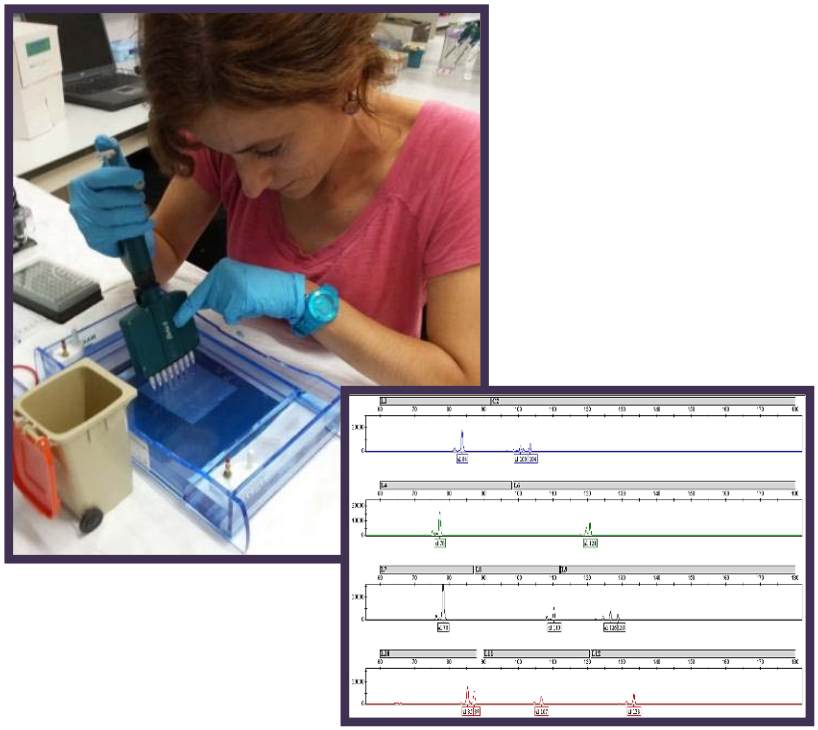
In this context, the main objective of this study was to analyze and to determine the existence of QTLs affecting the most frequent and important skeletal deformities in gilthead seabream, which could be useful to minimize these deformities prevalence when being considered within a specific genetic breeding program.

To get that objective, the following secondary objectives were established:

1- To develop a set of multiplex PCRs containing the highest number of gilthead seabream specific microsatellite markers, to cover the highest proportion of linkage groups of the genetic map of this species, so it could be used as an efficient tool to identify QTLs. As well as to evaluate and to validate its proper performance.

2- To analyze the prevalence of four skeletal deformities (lack of operculum, lordosis, vertebral fussion and LSK complex), and to determine if they show a correlation with the family structure, in different gilthead seabream populations. In order to confirm the genetic origin of these deformities and to identify a family structure that could facilitate the search for QTLs.

3- To genotype, by using the set of multiplex PCRs developed, the families showing a significant association with the analyzed deformities to identify and localize QTLs associated to these deformities.



2. A set of 13 multiplex PCRs of specific microsatellite markers as a tool for QTL detection in gilthead seabream (*Sparus aurata* L.)

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Oral comunication. 4º Congreso Nacional de Acuicultura. Enero, 2013. Puerto Montt. Chile

Poster. Aquaculture International Conference. Noviembre de 2013. Gran Canaria. España.

2.1. Abstract

The growth and consolidation of the gilthead seabream (*Sparus aurata* L.) industry require improvement based on permanent and cumulative aspects, such as those derived from genetic breeding programs. Marker Assisted Selection (MAS) by Quantitative Trait Loci (QTL) can be usefully implemented with the appropriate tools. In this study, 138 microsatellite markers from the genetic map of gilthead seabream were redesigned to be amplified under the same PCR conditions. A final set of 13 multiplex PCRs (named ReM*sa*) with 106 of these markers was developed to cover 100% of the linkage groups. These effective multiplex PCRs enable to optimize QTL searching with a critical reduction in costs and errors. Results showed that the mean value of the number of alleles for 106 markers was 6.9. The mean observed heterozygosity ranged from 0.53 to 0.86, and 74.5% of markers were highly informative according to their polymorphic information content. The correct inheritance and segregation of alleles of each locus was confirmed after genotyping 62 individuals of a full-sib family by these multiplex PCRs. Additionally, genetic features of the 20 microsatellite markers that worked correctly but were not included in any multiplex PCR are also reported to provide geneticists with the possibility of including them in more comprehensive screening studies.

2.2. Introduction

Gilthead seabream (*Sparus aurata* L.) is one of the most important species in Mediterranean aquaculture, reaching an annual production of 151,346 tons in 2011 (APROMAR, 2012). This growth and industry consolidation require greater efficiency production. Although scant attention has been paid to genetic aspects to date, it should be noted that selective breeding is continuous, permanent and accumulative; and could be incorporated into the production line. Thus, selective breeding is widely recognized as a key factor in the development of resource-efficient, sustainable and profitable aquaculture productions. In gilthead seabream, some genetic parameters have been reported for presence/absence of deformities (Astorga *et al*., 2008), resistance to fish pasteurellosis (Antonello *et al*., 2009), growth (Knibb *et al*., 2009a) and carcass and flesh composition traits that have important economic interest under industry conditions (Navarro *et al*., 2009b). To facilitate selection based on individual information rather than in phenotypes of sibs, genetic marker information such as Quantitative Trait Loci (QTL) may be implemented through Gene or Marker Assisted Selection (GAS and MAS, respectively) (Rye, 2012). GAS and MAS are increasingly important tools in genetic animal breeding; however, they only will be profitable if they form part of a classical breeding program (Pérez-Enciso and Toro, 2007). In gilthead seabream, by combining the use of physical tagging and genetic identification, it is possible to implement a genetic improvement program without interfering with the production system (Navarro *et al*., 2009a). This model has been developed at national scale in Spain under a research project PROGENSA®, funded by Spanish Ministry of Environment, Rural and Marine (MARM) (Afonso *et al*., 2012). For an efficient QTL detection, a genetic linkage map with a good level of saturation of molecular markers, suitable phenotypic data and an appropriate statistical-genetic model are needed (Lynch and Walsh, 1998). In gilthead

seabream, a first-generation genetic linkage map based on 204 microsatellite markers and 26 linkage groups (LG) has been published by Franch *et al*. (2006). Microsatellites are probably the most useful molecular markers for medium-high density maps, because they are highly polymorphic, codominant, widely distributed throughout the genomes, and readily assayable using polymerase chain reaction (PCR) (Bouza *et al*., 2007). For a given QTL, the likelihood of success and mapping resolution depend on the number of loci screened and the magnitude of their effect on the trait of interest (Chistiakov *et al*., 2006). In this species, a few QTLs associated with commercial traits have been reported. Two QTLs were found in LG13 and LG21 for resistance to pasteurellosis using 151 microsatellites (Massault *et al*., 2011). Boulton *et al*. (2011) detected a QTL that is significant at the genome–wide level for morphometric traits and two suggestive QTLs for stress response to confinement with 56 microsatellites. Three other traits of economic importance in aquaculture, such as growth, sex determination and morphology, are influenced in this species by four, six and two QTLs, respectively, detected using 74 microsatellites (Loukovitis *et al*., 2011, 2012, 2013). Therefore, in the QTL search, a considerable number of microsatellites are required to cover the largest possible number of loci and a lot of animals have to be genotyped. However, none of these QTL was detected by multiplex PCR, which is well known to be a highly effective tool to reduce cost per reaction and minimize genotyping errors by reducing steps and introducing automation during the sample analysis process. It should then allow the optimization of QTL detection. In fact, Navarro *et al*. (2008) reported that the cost of genotyping by a multiplex PCR based on 10 microsatellite markers is no more than one-sixth that it performed by single reactions loaded in a just one run in the automatic sequencer. Many multiplex PCRs have been developed in gilthead seabream to successfully determine family relationships (Launey *et al*., 2003; Brown *et al*., 2005; Navarro *et al*., 2008 ; Porta *et al*., 2010; Borrell *et al*., 2011). Moreover, Vogiatzi *et al*. (2011) proposed five multiplex PCRs with 32 microsatellites found in coding sequences while Lee-Montero *et al*. (2013)

developed two multiplex PCR reactions (SMsa1 and SMsa2) and proposed them as an international panel in this species. However, no sets of multiplex PCR assays from microsatellites of the gilthead seabream linkage map, appropriate for QTL search in this species, have been published.

The main objective of this study was to develop a set of multiplex PCR essays from a wide spectrum of specific microsatellite markers to cover the highest proportion of LG in the gilthead seabream genetic map, which would constitute a powerful tool to search and identify QTLs in this species. A secondary objective was to evaluate it and validate that it works correctly.

2.3. Material and Methods

2.3.1. Samples and DNA extraction

A total of 16 gilthead seabream from three Spanish industrial broodstock belonging to the PROGENSA® breeding program (Afonso *et al*., 2012) were analyzed to study the genetic variability and to carry out an initial genotyping reliability evaluation of the microsatellite markers (control samples). From these samples, four individuals were used to optimize the multiplex PCR reactions (testing samples). Moreover, a full-sib family of 60 offspring and their parents (validation samples) were used to check the correct segregation of alleles and to revaluate the genotyping reliability in each microsatellite marker. DNA was extracted from fin tissue previously preserved in absolute ethanol at room temperature, using the DNAeasy kit (QIAGEN®) and then kept at -20°C under instructions provided by the manufacturer. DNA quality and quantity were determined using a NanoDrop 1000 spectrophotometer v.3.7 (Thermo Fisher Scientific, Wilmington, U.S.A.). DNA integrity was checked on a 1% agarose gel (8 $V \cdot cm^{-1}$) by ethidium bromide staining (0.5 $\mu g \cdot \mu l^{-1}$) and analyzed with Quantity One© Software (Bio-Rad Laboratories, Hemel Hempstead Hertfordshire, UK), using Lambda HindIII as a molecular weight marker.

2.3.2. Multiplex PCRs

Redesigned primers

Out of all the published microsatellites in the genetic map of gilthead seabream (Franch *et al.*, 2006), 138 primer pairs (Table 1) were redesigned to amplify markers using the same PCR conditions (Lee-Montero *et al*., 2013). Primer characteristics were chosen to optimize the effectiveness of the multiplex reaction (Sánchez *et al*., 2003).

Table 1. Description of microsatellite marker used in this study. Internal code (IC), Loci names, loci accession number in GenBank, fluorochromes and redesigned primer sequences

IC	Names	Accession	Forward primer sequence (5′→3′)	Reverse primer sequence (5′→3′)
Fluorochrome 5'6-FAM				
A 1	CId-35-H	DQ851311	AGTCTTACAGAAAATGGTTTCCACA	gtttAGAGTGCAGTGGCTGTCTTCTTAT
A 2	48EP	DQ851237	ACCACCTACAGTTTTCTCTGCTG	GCGTGTAAAAGTATGAACTCTTCAG
A 3	Bd-75-H	DQ851273	TGACGTCAGGGTCATAAAGC	gtttCCTACGCTCTAGCCACGTGAT
B 1	CId-24-H	DQ851306	AGATCCCTCACTGCAGCAAA	gtttAGTCTGCATGTTTATATGAGAGCAA
B 2	CId-89-H	DQ851318	GATCTTAATATATTGCATGGACGTG	GCCGAAGTATATGAAAGGAGTGTAA
B 3	Gt57	DQ851386	CCTGACTCTTACCCCAGTCG	gtttAATTACTCCTGTGCTGCATTCTG
C 1	DId-14-H	DQ851338	CCTTGAAAAGGACACTCACTGATAG	gtttATCCCGCTGTCCGAATGA
C 2	Fd-48-F	DQ851374	ATTACAGCCAGAGGACTGGAGAG	gttTCAGAGAGAGTGTTCTTGTGTATCTG
C 3	EId-39-T	DQ851367	GAACTGTGTGAGAGAGGACAGTTG	GAAGGAAAAATTCTGATTGGAGTTC
C 4	Saimbb16	AJ418652*	ACCTGTTTCTTTGATTTGTTGC	gtttCATAACAGCAGGATAGGTCAGACT
D 1	Saimbb3	AJ418671*	CTTCCCGGATTTGCTGAC	GTCAGCCGAGGAAAAGGA
D 2	Saimbb26	AJ418656*	CAATGGCAAAAGCAATGG	gtttAAGTGACGACGCAGTGTGA
D 3	Dt93	DQ851348	TGGATGTGTATGCGTGTGC	GCCTTGTGGAGTGCTGGTAT
D 4	P3	DQ851284	GAGGGGACACGTAAATTTGG	gtttAACAACAGTCAAATGCTGAGTTGT
E 1	Bd-68-T	DQ851270	AGGGGCGTTTCAGGTTCT	gtttAGGTGAGTCCGTCAATCAGTGTAT
E 2	Bd-54-F	DQ851266	GATGAACTATCAATACTCTGCTTACTCTC	gtttCGGTAACAAATGCAAAAGCTGTAAT
E 3	Fd-81-F	DQ851377	GGCTGGACATGCTTTCATAGAT	gtttAGCGCTTGAAGATAAAACAACTCT
F 1	Bt96	DQ851295	TCCAGCTTGGGATCAATAAAGTA	GCTAGTTTTCCTGAAACAGTCATTT
F 2	CId-26-H	DQ851307	ATGTGAAACAGAGTGGAAGAAAAAC	gtttAGACGACAGACAAAGACCACAGT
G 1	Dd-10-H	DQ851322	AACACTGTTCTTTCTTCAGGACACT	GGCAAGCTGTCGTTGTGTT
G 2	CId-03-F	DQ851301	ATGACTAAACATCACGTTCATGGAT	gtttAGCTCATGGCTAACTGTGTACTTTT
G 3	Hd-23-T	DQ851391	TCTAACTTTCTTTGAGGTCCCTCTT	GAGGACAAGCTGAAGTACGAGTC
H 1	P19	DQ851285	GGATCTGCAATATCCACAGTCAG	gtttCTGGATGCAAAGCTTTCTCTG
H 2	Dd-56-F	DQ851326	GTAGTGCTGGAACAAGACCAAAAG	gtttCGTCATCCAAAAATCTTTATCTCTC
H 3	C67b	DQ851296	CCTGGTTATCATCTGTCGTTGG	gttTGTTGTTTGCTGTCTGATTGTAGTG
I 1	CId-31-T	DQ851309	GGAGAACGGGCACACTGTATATC	gtTTGTCTTCCACCTGTAATACCATCT
I 2	Fd-78-H	DQ851375	CATTTACAGCTAATGCTCAGAGAAA	GTGACTGAAGACAGGTATGAGCAC
J 1	P96	DQ851292	CGCAATTAGAAGTAGGAGACTGG	gttTGCCAGATGCAGGATGTAAG
J 2	DId-24-T	DQ851343	AGAGAGCTTCACTGACCCTGAG	GCTGAAAACGTGGTTTTGTTAGATA
J 3	Bd-06-H	DQ851259	GGCATCAGCAGCAACCAG	gtttAGGTAAATACCACCGCTGTCAC
K 1	Ad-29-H	DQ851242	AGGAAACATTCCAAGACAGCAC	gttTGTGTATTGCAAACTTCAGTGATTG
K 2	Dd-84-H	DQ851331	GGGTGATGAATTCGTTGGAC	gttTCCTCTTTCAGACACATTACTCACA
K 3	EId-13-H	DQ851361	CTGACAAATCTGAAGATCTGAATACTG	GATCCTCTGCTCATCCATCTG
L 1	BId-39-T	DQ851282	ACGGATGCGGGTAAATGA	gtttAAGACACAAAGTTACACCATGTGAG
L 2	Id-42-H	DQ851408	AGTATCACGTGACGGGTTATGTTTT	gtttAAAAGGAATTACGGTGGAAACAC
L 3	DId-18-T	DQ851340	ACCACACATGAAATACACACAGAGA	gtttATGTGGCTTTGCTTGTTGTTT
M 1	BId-36-H	DQ851281	GCACTCAGCCATAAACAACAC	GTCCTCTATTTTTCTGCTTTGTGAT
M 2	Gd-60-T	DQ851381	GACATAGTGAAGATAGCCATCCAGA	gtttAGAACAGGCAGACAACAGAGACTAT
Fluorochrome 5'NED				
A 4	Hd-46-T	DQ851397	CCTTCCCCTGACATGTTTTG	gtttCAGGGTGTAACACATACTGACTGAT
A 5	Bt-14-F	DQ851293	AGCCGAGTACTTCTACTCCTCTGAT	gtttAGTGAGGGCGGACAGATAAAG
A 6	B6b	DQ851256	TTATCACAGGACCATTAACGTATAGAG	gtttCTATCAGCCCCATCTTTTCAATA
B 4	EId-05-F	DQ851356	GCTCTACTTGAGCAGTCTTTGAGC	gtttCTCGCTGAGTGATGCAGCTATAC
B 5	EId-37-T	DQ851365	TATTCAGTGACAATTTGTGCTCATC	gttTCTTAACCCTTGACAAGTCTCTGA
B 6	Dt23	DQ851346	ACACAACACACGATTACAGCAGA	gttTCCATGTGAGATGTCACTCTATTTC
C 5	Hd-10-F	DQ851389	CCATCAATCAGCGAACCAAG	gtttCTCAAAACCGACAGGACGAC
C 6	Id-14-H	DQ851404	TCGTTCCATTATCTGGATGTAACTT	gtttCTCCTAATGTGGTTCCCTTTTACTC
C 7	DId-04-T	DQ851334	GAGATTACACCACAAAACGATCAAT	gttTGCTGTACCAGTGGTTGTTATATCC
D 5	Gd-46-F	DQ851380	TGATGTGTGTCATATCAATTCACAG	gtttAGGGAATTATGCAGAGGGTTTT
D 6	Fd-42-T	DQ851372	TCCAAGAGTGTGAGCAGTCTAATCT	gtttCAGGTCCAACCTGCTATCAATTA
D 7	SAGT41a	Y17263***	GTCAAAGACAGATGGAGCTGGT	GGGTCACATCAGTCTGCACTT
E 4	BId-18-F	DQ851280	AGTGATGCGCTCTGGGTTTTA	GTCTCTCAGCCTTTGAAGTGTTATC
E 5	Ef6	AJ418667*	AGAACAAATGAACTCAGCAACTTTC	gtttAGTCTCCTGTGTTTATGATGACTGC
E 6	Sai21	AY322112**	TCAGAGCAGAGCTGTGATTGTAT	gtttACCGAAGCTGATTGTTAGTGTGAGT
F 3	Saimbb25	AJ418675*	AGGAAGATGACAGTGGAAACATTAG	gtttCCCCTACCTTTTCTCTTCTAACTCA
G 4	CId-14-F	DQ851304	ATGTGAGTGCACACGTGGAG	gtttCCTGTCAGGTATATGAGAAGAACTAATG
G 5	Gd-67-H	DQ851383	CAAGCTCAGGTGTAGCATTAGTCTT	gtttAACTGGTTAGGAGTGTTGCTAAATG
H 4	B26b	DQ851254	TTGACTTGACCCAATATCTCACTCT	gttTCTCAGGTGACTAACAAGTGAACAG
H 5	Hd-15-H	DQ851390	CTCTCTCATGCGCACTTTCTT	gtttCACTCCCTCTGATTTATGAGATGAT
I 3	EId-06-F	DQ851357	CAAGCATGTGAAAGTATGATTTGTG	gtttCCCTCATAGCCTTGTAGCTCAG
I 4	Bd-08-F	DQ851260	GAGACAGCAGGTCGCTCATC	GACAGAGACTAAAGCTCGACGAA
J 7	DId-12-F	DQ851337	GATGAGTTAAGAGCGTGTGTTTGT	GTTTGCACATCAAAGCCATATAGA
J 8	Bd-48-T	DQ851265	CAGGGAAACAACACAATGCTG	gtTTCCGACAGGTTGTACATATATCAG
J 9	Fd-46-T	DQ851373	ATTTGACTTGATGATCAGCTGTG	gTTTTCTGGAGTATGACTGAAAGACA
K 7	CId-65-H	DQ851315	ATTTATACCACAGCTCAATGACACC	gtTTCCTTCTCGTTTATGTAAGTCTGC
K 8	SAI15	AY322110**	CTGTCTTTCTGTCCCTCACACTTAT	gtttCTCAGTTGAAGCATTCTGTAAAGTC
9	Bd-86-T	DQ851275	ACTCACTGGGGATCAGGAAAC	gtttCACTGCAGCCTGTGTCAGTTAT
L 7	Dd-57-T	DQ851327	ATCATTGTTCAGATAATGGGACAC	gtTTCAAAATCTTTTGGTCTGTGC
L 8	Hd-01-F	DQ851388	GTCCTTATAATGTGCGCAATAACTC	gtttAAGTCTGGAGGACATCAGTGTAGAT
L 9	DId-03-T	DQ851333	ATTTATGTCATCTCCCTTTCTCCA	gtttACTGTGGTCATGTTTTTCCAGTC
M 5	Ct27	DQ851320	GAGACAGAGAGGAAGAAAAAGGATT	gtttCAATGCTACAAGCTGCCTCAG

Table 1. Continued

M 6	Fd10	DQ851370	ACCACTCAACAATGACAGGCTAGT	GGGATCTGAAACATGAGAGAAGAC
M 7	Saimbb20	AJ418655*	GCTGTCTCTACGCTTTCAGCTC	gtttACTTTGCATTTCCCTGCCTAT
Fluorochrome 5'PET				
A 7	Gt82	DQ851387	ATGCTGAGGTTGCTCTTCATCT	gtttAGAGTGAGTGAGAGTGTGAAAATTG
A 8	C90b	DQ851299	ACAGAGTCAAGCAGCGGATAAC	gtttCGACCTGCTGAGGAGAACA
A 9	Ad-07-H	DQ851247	AGATTACTTAGTCCTAACTGTGGTAGTGA	gtttCTTCTTCTGTCTGTCCTTTCACG
B 7	BId-15-F	DQ851279	AAGAGAGGACTGTGAGTGTCTGTG	gtttAGCTAAAAGCTACTGGGAGAGAAAG
B 8	Hd-33-F	DQ851394	TGAGTCTACAAAGCCAGAACCA	gttTAAGAGAATAAAGCTCTGCCTGTGC
B 9	Dd-63-H	DQ851328	CAAGACAATTCTAGGCCATTATACG	gtttAAGGGAAAACTATCAGGGCTCTT
C 8	P48	DQ851288	CCGAGTCGCTGTTAGTGGA	gtttATACAGCAATTCATTTACACACCAC
C 9	Id-39-H	DQ851407	ACCAAACAGACAGCTGAAGGTTAC	gtttACATTTAGAAAAAGGGAGGATCG
C 10	EId-38-F	DQ851366	TCTGAATAAAACATTGTCTGCAGTG	gtttCTCAGACGGAGTATTTTTGGAACA
D 8	EId-17-T	DQ851363	GTAGCATCGATTCTCAGCAAAC	GGCAAGATGTTCCACCTCCT
D 9	CId-44-T	DQ851312	CTTTGACTCCTGACCTCTTAATCC	gtttCAGAGTCAGTTCCTTTCCTCTCTGT
D 10	SAGT26	Y17266***	GGTTCAGTTTGGAGTATGTTTGAA	gttTCAGCTATAGAGATGTCTGTGTTCTC
E 7	P33	DQ851287	ACTGACGCTGGCTGAGATTT	gtttAACATCACGATGGGCACAA
E 8	Hd-70-H	DQ851399	TACACACACACTCTCTCTCTTCAGG	gtttATGGAAGAGGCAGGAATCTTTT
F 4	P54	DQ851289	TGTCTCTCTATTGTCCTCTCTCCTC	gttTGCACCTACAGCGGCATC
F 5	Id-11-H	DQ851402	GAATGTCTGTAATTGTTTGCGTGT	GGTCAATTCAACATATCATCCATC
F 6	Hd-25-F	DQ851392	AACGGTGTCCTCTATTTCCATTATT	GCAGGAATTATCTATGTCTGCCTAA
G 6	CId-52-T	DQ851314	GTAAATCCAACAATGTAGAGTGACG	gttTCCTATTAACATGAATGAGGGTGTT
G 7	EId-11-H	DQ851360	TGAAAATCACACAACTGGATGC	gtttAGAGAGCAGAAGAAGAGACGAGTTA
H 6	DId-09-F	DQ851336	TATTAGCGCGTAAATGTAGGTTCTT	gtttCTGACAAGAGGCTGACAGGAG
H 7	DId-07-H	DQ851335	CGACAACAGTCAGGAGTAACAGAT	GCCTGGTCACCCTGAAAC
I 5	CId-71-T	DQ851316	GCGGTGAAGACTACCTTGAATACTA	GTGTGTTGACAGAAAATGTACAGGA
I 6	Bd-58-F	DQ851267	GGGATAATAACCTGTAAGTGAGCAA	gtttCAATGTTCCCACTCACATCC
J 10	CId-11-T	DQ851303	CGTGTGAAGTTGTGTTAAGTTGTG	GATGGACACGGTGGTTTAACA
J 11	Ad-54-F	DQ851243	GTGCATTCAATTGACAGAAAGAGAT	gtttCAACAGTGTCATTGTGCTGGAG
J 12	Bd-72-H	DQ851272	AGATGCTGAGTCCATAAACCAGATT	gtttACAATCTACTGCTCTAATGGCAATG
K 10	Bd-77-F	DQ851274	CAGTGACTATCACACAGCACAGATA	GGCCAGCACACATCTCACT
K 11	CId-48-F	DQ851313	TGAATCTGACGTCCTTTACGG	gtttAAGCATGTTTCACGGGTACG
K 12	Ad-66-F	DQ851245	AGAACCTGAACGAAGCTGTTG	gttTCGGACACACACTGTTTACCTTAT
L 10	Gd-78-F	DQ851385	ATAGATCCTGTAACACGCATACACA	gtttACCGAGGGAATACCATCACAG
L 11	DId-16-F	DQ851339	GTTGTAGATCGGAGTGTGATAACG	gttTGCTCCGAGTAAGCCATATGTA
L 12	Hd-49-H	DQ851398	CGAAATGTGAGTAAACAGTCAAATG	gtttCAAGGAGACAGGTAATGCACTG
M 8	Bd-29-H	DQ851263	CACATGAGCACATTTGCATTTATAC	gtttCTCCCATGACAGCCATCTC
M 9	Hd-45-F	DQ851396	ACATACACCAGGCAGGCATAG	GTTGGAAATAGCATTTGGGACTT
Fluorochrome 5'VIC				
A 10	CId-21-F	DQ851305	GCCACATGTCCACTCCAAGT	gttTATTGAGTGCTGTGAAAGAAGAGG
A 11	Dd-16-T	DQ851323	ACACACATTTAGGGCACCATATC	gtttAGATTGCGGAAGAAGTATGAGAGAC
A 12	Ad-12-F	DQ851239	GAGCCATTTCAGTTTTTATTGGTTC	GCCATCCAGCTGGTCCTC
B 10	C94b	DQ851300	TCATGGAGATCAGGAGAATACTACA	gtttAGTTTTGTCCCCAATGTGATTAAC
B 11	Bd-71-F	DQ851271	GCCCTCACTAATGAAATGATGTTT	GTGCAGGAATGTGAACTATGTTTCT
C 11	Saimbb1	AJ418672*	AGCCAATACTTACCTCCCTTAGGAT	gtttCTTCCCTGCCACAGAAGC
C 12	Dt47	DQ851347	TGCCTCTCTTTCACTCACTTCTC	GCACATTGCTCCACACAGAG
D 11	CId-29-T	DQ851308	GCTCAGCACTACTTTAGTGTTTTGG	gttCTGCAGGGAGGAAACAAGAC
D 12	EId-41-H	DQ851368	GATTCCCATCGTTTGTAAAGTTGAT	GAGGACGACCTCAACCTCATAC
D 13	Saimbb18	AJ418632*	GTCAGTGATGACATACAGATCACCT	gtttAGTTCAACTTAAAAAGGGCAGAGTT
E 9	Fd-92-H	DQ851379	TAGAGAAAGGCATTTTGTCAATGAG	GCATCAGACAGACGGGTACAGT
E 10	Simbb9	AJ418665*	AGGATGACTGTGTTCAAACGTATTT	gTTTGAGAGCTGTAAAAGTGCTGTAT
F 7	Hd-77-F	DQ851400	CATGTGTTGTTTGTTTGTCAGAGTG	gtttCAACATACATGCATACTGAGGAAAG
F 8	Dd-91-F	DQ851332	GCTAACATTACACTGGGAACCTAAA	gtttAAGCCAATTAGGCGAAATGAG
G 8	DId-30b-T	DQ851344	GTTTCCCTGTGGGTTCACAAT	GTGTGTGTGTGTGAATTTAAGAGGA
G 9	DId-31-H	DQ851345	AACAAACAGCGTTGTCTCAGAAC	GAAACCTAAGGCCTCATTTGG
H 8	P60	DQ851290	ATGCTGACATAACACAATGTAGCTC	GAGAAGTGAGGGGATACCTGAG
H 9	Bd-61-H	DQ851268	GATTGGATGGGAGGACTGAAC	gtttATGCACAAACATGCAAGCATAC
I 7	Fd-90-H	DQ851378	CAGACTGAGGAGATGGAGGAGAC	gtttAGACTCTGTGTTTGGTACAGCAGAT
I 8	B13b	DQ851252	ATGACAGTGTTTGAGCTCAGTGTTA	gtttCCTGCATTCCAGCTTCAGAT
I 9	At37	DQ851250	GGTTAGGGTAATCAGAAATGCAATG	gtttCTTCTCCAGTCACGATCAATAAAG
J 4	C80b	DQ851298	TCCGCTGAGCTGATGTGAT	GGATCCGACAGGTGTCACAA
J 5	B18b	DQ851253	CTGACCTGATTTCTTTGTTGTTGT	gttTCACAGAACTTGAACAGCTTGTATT
J 6	Ef7b	AJ418660*	GTTCGCACACCCACAATCT	gtttACAATGCAAACACTGCAACTAAG
K 4	EId-08-H	DQ851358	CTGTTGACGTGGTACTGAATACATC	GGAAAGTTCTGAAAGAATAAGAACC
K 5	Hd-91-F	DQ851401	TCATAATGGCTTGATCTTTACACTG	gtttCTCAGCACGGAACTCAAGACATA
K 6	B82b	DQ851257	GCAGAGCAGTGGAAGCAAG	gttTCCCCTCTCCTGTGTCTTTTACTA
L 4	Bt-34-H	DQ851294	AAAATTCAGCTCGGAGGATAGG	gttTGTTATCCTCAGTCTCAGTATCATCAG
L 5	Fd14	DQ851371	ACAGATGGTGTGTGACAAATTCA	gttTGGTTGGCTGGCTCCTTATTA
L 6	Ed-31-T	DQ851351	GGCATTTTAGAAAGAGCCAAATG	gttTGGAGATGCTATTGTTCTGTCTG
M 3	BId-04-F	DQ851276	GATCTCATTATGACGGATCATTAGC	gtttATCTTTGTCCGCATGTTTCAC
M 4	DId-22-F	DQ851342	GGTCAGATAGTTCAGTCATGGATTC	gtttCTCCCCTCTACCTCCCAATAAGT

All markers were originally described by Franch *et al*. (2006), except those indicated with an asterisk (Argyrokastritis *et al*., 2002), two asterisks (Brown *et al*., 2005) or three (Batargias *et al*., 1999).

The chosen amplicon length ranged from 70 to 200 base pairs (bp), the existence of flanking DNA sequences free from interfering polymorphisms was considered, GC content stood at 35–60% and hairpin or secondary structures were avoided using the software found on-line at Integrated DNA Technologies (http://www.idtdna.com/Scitools/Applications/mFold/). Primers were named with the same internal code used in Lee-Montero *et al*. (2013) in order to facilitate understanding of this work.

PCR reaction and microsatellites evaluation

Each microsatellite marker was tested in a single PCR using the control samples in order to check its correct amplification, allele size range and banding pattern. The PCR conditions consisted of an initial denaturation at 95°C for 10 min, followed by 28 cycles of 94°C for 30s, 60°C for 1min and 65°C for 1 min, with a final extension of 65°C for 60 min. Reactions were carried out in a final volume of 12.5µl with the following component concentrations: 1X GeneAmp PCR Buffer II (100mM Tris–HCl pH 8.3, 500mM KCl) (Applied Biosystem®, USA), 3mM $MgCl_2$, 0.2mM of each dNTP, 0.04 units μl^{-1} AmpliTaq Gold DNA polymerase (Applied Biosystem®), 2.4-6.4 $ng \cdot \mu l^{-1}$ of DNA template, and 0.2µM of each primer. Before running PCR fragments in the automatic sequencer, an aliquot was checked on 2% agarose gel for 30 min ($8V \cdot cm^{-1}$) to test the appropriate amplification of each marker. Later, 1µl of each reaction product (diluted at 75% with Milli-Q water) was mixed with 9.75 µl of Hi-Di formamide and 0.25 µl of *GeneScan LIZ 500* (Applied Biosystem®, USA) molecular weight marker, and loaded in an *ABI Prism® 3130XL automatic sequencer* (Applied Biosystem®, USA) with 16 capillaries using POP-7 polymer and run conditions of 60°C, 3000 V, 1500 s. The reaction products of each single PCR assay were initially loaded in groups of three markers taking into account their fluorochromes, allelic ranges and the intensity of the band on agarose gel. Electropherograms were *analyzed* using the *GeneMapper* (v.3.7) software (Applied Biosystem®, USA). The genotyping reliability of each microsatellite was evaluated according to the *GremmProtocol* (Lee-Montero *et al*., 2013). This method

classifies genotyping errors or potential errors into four types, according to their peak scoring (inadequate amplification, long allele dropout and unclear banding pattern) and allele calling (intermediate alleles). Since, the reliability of each microsatellite genotyping is evaluated by quantification of these errors or potential errors in samples of each study and scored as 1 (ambiguous genotypes in more than 30% of the samples), 2 (ambiguous genotypes in 30% or less of the samples) or 3 (unambiguous genotypes in 100% of the samples).

Multiplex PCRs optimization

Monomorphic markers or markers without amplification were discarded from the multiplex reactions. For the remaining markers, the maximum number of multiplex PCRs were designed including the higher possible number of markers in each one taking into account their allele size range and assigned labelling fluorochrome (in order to avoid overlapping). These multiplex PCRs were named ReMsa (Redesigned Multiplex *Sparus aurata*) Design and optimization were performed for each multiplex reaction sequentially one by one in such a way that when the amplification of a marker failed in one multiplex PCR, this marker was included in the next one. The initial concentration of each primer was 0.2 μM for each multiplex PCR and it was subsequently modified to obtain peak heights between 600 and 3000 RFU for each microsatellite marker as described Navarro *et al.* (2008). Each set-up was performed on the testing samples for each multiplex reaction. PCR conditions were the same as those used in the initial single PCR. Again, an aliquot was checked on 2% agarose gel for 30 min ($8V \cdot cm^{-1}$), to verify the appropriate amplification of the multiplex amplicons. The automatic sequencer conditions were also the same as those used in the single PCR. The reaction product was not diluted, except in ReM*sa*1, ReM*sa*6 and ReM*sa*8 in which 50% of PCR product dilution was needed in order to improve the peak scoring. Electropherograms were analyzed using the *GeneMapper* (v.3.7) software and a kit of bin set was created for each multiplex PCR.

The observed heterozygosity (Ho), expected heterozygosity (He) and the Polymorphic Information Content (PIC) of each microsatellite marker, and the Combined Non-Exclusion Probability for the parent pair and second parent options (Jamieson and Taylor 1997) of each multiplex assay were estimated using *Cervus* (v 3.0.3) (Kalinowski *et al*., 2007). Markers were sorted according to their PIC value as highly informative (PIC≥0.5), reasonably informative (0.5>PIC>0.25) and slightly informative (PIC≤0.25) (Botstein *et al*., 1980).

2.3.3. Multiplex PCRs validation and genotyping reliability revaluation

Validation samples were genotyped with each multiplex PCR optimized in this study. These genotypes were used to analyze the inheritance and segregation of alleles. Offspring genotypes were checked to verify that one allele matched one breeder and another allele matched with another breeder. False homozygotes in one parent and several descendents allowed to identify null alleles. Moreover, these genotypes were also used to check the values of the genotyping reliability of each microsatellite according to the *GremmProtocol* (Lee-Montero *et al*., 2013).

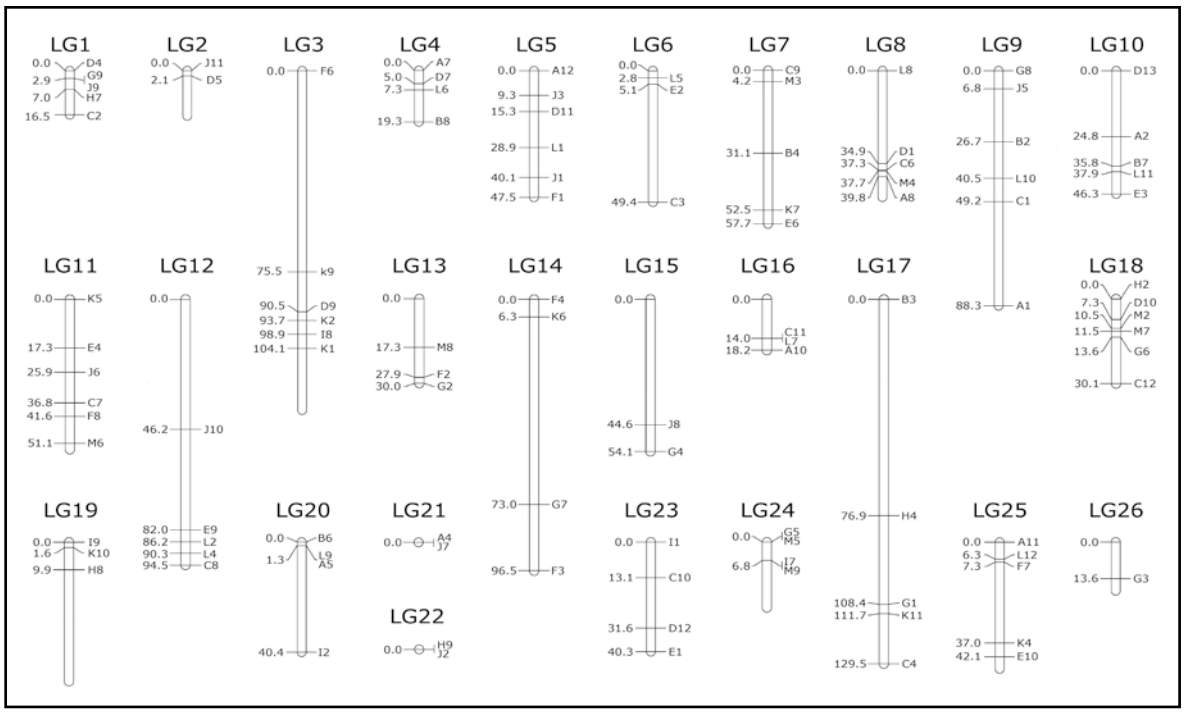


Figure 5. Genetic distances between markers used in this study based in 26 linkage groups from gilthead seabream genetic map described by Franch *et al*., (2006)

2.4. Results

2.4.1. Multiplex PCRs

After initial evaluation, 10 of the 138 microsatellite markers (A3, B9, B11, C5, D8, E7, H3, J4, J12, K12) did not work individually and two were monomorphic (D3, M1). Of the remaining 126 markers, 106 markers were combined in 13 final multiplex PCRs named ReM*sa*1- ReM*sa* 13. When a marker failed to amplify in a multiplex PCR reaction, it was included in another one, thus the multiplex PCR design and optimization were performed sequentially. Only two markers, C3 and D10, did not work in the first multiplex PCR in which they were tested (ReM*sa*3 and ReM*sa*5**,** respectively), but they worked in another multiplex PCR (ReM*sa*13). The remaining 20 markers, named alternative microsatellite markers, were dismissed from multiplex PCR assays because of their allele size range or labelling fluorochrome (B1, B5, D6, E5, E8, F5, H1, H5, H6, I3, I4, I6, K3, K8 and L3), or because they did not work in any of the multiplex PCR in which they were tested (A6, A9, B10, D2 and I5). The final number of markers of each multiplex PCR and their concentration, observed and expected heterozygosity, polymorphism information content and the genotyping reliability evaluation of all markers are shown in Table 2. The number of markers in the 13 multiplex PCRs ranged from 6 to 11. The mean value of number of alleles for these markers was 6.9 (from 2 to 21). Ho ranged from 0.125 to 0.999, and He ranged from 0.121 to 0.978. Most markers (74.5%) were highly informative according to their PIC value, 21.7% were reasonably informative and 3.8% were slightly informative. Mean values and combined non-exclusion probabilities of each multiplex PCR are shown in Table 3. Out of 1241.9 cM from the first genetic linkage map of gilthead seabream, this set of multiplex covers a total of 990.1 cM. The genetic distances between markers are showed in Fig. 5.

Table 2. Genetic information of each microsatellite marker of 13 multiplex PCRs (ReM*sa*) and alternative microsatellite markers on 16 gilthead seabream samples (control samples): Internal code (IC), locus, fluorochrome (Fl), linkage group (LG), type of nucleotide motif, number of alleles (NA), allele size range (bp), primer concentration (µM), observed (Ho) and expected heterozygosity (He), polymorphism information content (PIC) and genotyping reliability (GR) revaluated by GremmProtocol

IC	Fl	LG	Nucleotide motif	NA	bp	µM	Ho	He	PIC	GR
ReM*sa*1										
A1	6-FAM	9	(CA)10	5	68-78	0.1	0.571	0.529	0.483	3
A2	6-FAM	10	(CA)12	2	82-98	0.1	0.25	0.484	0.359	3
H2	6-FAM	18	(GT)28	15	106-190	0.1	0.733	0.947	0.909	3
A4	NED	21	(GT)12	3	87-97	0.1	0.5	0.524	0.428	3
A5	NED	20	(CA)15	4	104-110	0.03	0.813	0.696	0.608	3
M5	NED	24	(TAGA)15	13	134-204	0.1	0.999	0.897	0.855	3
A7	PET	4	(CT)13	3	86-92	0.1	0.2	0.297	0.26	3
A8	PET	8	(AC)30	10	94-118	0.1	0.733	0.89	0.844	3
A10	VIC	16	(CA)16	3	83-93	0.1	0.5	0.599	0.489	3
A12	VIC	5	(TG)15	10	128-172	0.1	0.875	0.851	0.804	3
ReM*sa*2										
B2	6-FAM	9	(AC)12	2	103-105	0.04	0.143	0.138	0.124	3
B3	6-FAM	17	(CT)34	7	116-139	0.04	0.467	0.589	0.544	3
M2	6-FAM	18	(CA)32	14	177-263	0.1	0.8	0.938	0.899	3*
B4	NED	7	(GT)15	13	78-111	0.03	0.999	0.94	0.902	2
B6	NED	20	(AGAT)13	12	138-200	0.09	0.999	0.921	0.877	3
B8	PET	4	(CA)27	8	101-137	0.1	0.667	0.8	0.62	3
F7	VIC	25	(TG)15	6	96-108	0.08	0.583	0.66	0.643	3
H9	VIC	22	(GAGG)6 (AC)2 (TG)2	11	140-186	0.1	0.938	0.883	0.84	3*
ReM*sa*3										
C1	6-FAM	9	(CA)12	7	75-103	0.1	0.375	0.819	0.763	3
C4	6-FAM	17	(CA)41	8	140-186	0.1	0.714	0.653	0.601	3
C7	NED	11	(CA)30	6	105-117	0.15	0.933	0.805	0.743	1
C8	PET	12	(TG)13	2	88-96	0.05	0.125	0.121	0.11	3
C10	PET	23	(AC)21 (CT)7	9	127-157	0.15	0.8	0.869	0.821	3
C11	VIC	16	(CTG)10	2	84-90	0.04	0.125	0.508	0.371	3
C12	VIC	18	(CT)19	6	119-133	0.08	0.813	0.722	0.652	3
K5	VIC	11	(AC)14	4	97-102	0.06	0.533	0.561	0.495	2
ReM*sa*4										
D1	6-FAM	8	(GCT)7	5	51-63	0.04	0.75	0.627	0.557	2
J2	6-FAM	22	(CA)21	9	89-115	0.07	0.8	0.857	0.807	3
D5	NED	2	(CA)8	5	70-88	0.07	0.313	0.383	0.348	3
D7	NED	4	(GT)35	19	102-171	0.1	0.875	0.966	0.932	2
D9	PET	3	(GT)14 (GA)4	7	115-140	0.07	0.75	0.756	0.687	3
D11	VIC	5	(GT)16	8	73-93	0.03	0.938	0.869	0.823	3
J6	VIC	11	(CA)8	2	132-136	0.03	0.25	0.315	0.258	1
I7	VIC	24	(GT)17	7	97-129	0.09	0.733	0.731	0.686	3
ReM*sa*5										
E1	6-FAM	23	(GT)10	5	77 -85	0.05	0.733	0.731	0.649	3
E2	6-FAM	6	(AC)13	3	90 -101	0.07	0.333	0.301	0.271	3
E3	6-FAM	10	(TG)30	12	129- 166	0.1	0.867	0.91	0.869	3
E4	NED	11	(AC)13	10	63 -90	0.08	0.867	0.867	0.819	3
E6	NED	7	(AC)41	21	99 -197	0.1	0.929	0.976	0.938	3
H7	PET	1	(GT)23	4	123- 129	0.08	0.625	0.732	0.654	3
E9	VIC	12	(GT)13	9	84- 105	0.04	0.733	0.876	0.83	3
E10	VIC	25	(ATA)9	3	114 -119	0.07	0.533	0.522	0.407	3

Table 2. Continued

M4	VIC	8	(GT)31	5	124- 134	0.15	0.571	0.741	0.671	3
ReMs*a*6										
F1	6-FAM	5	(TATC)13	12	80-138	0.06	0.833	0.929	0.892	3
D4	6-FAM	1	(TG)25	7	183- 205	0.15	0.8	0.816	0.76	3
F3	NED	14	(TAGA)10	9	98-134	0.05	0.923	0.887	0.863	3
F4	PET	14	(AC)14	7	72-86	0.08	0.667	0.763	0.701	3
F6	PET	3	(GT)15	6	124-142	0.09	0.938	0.7	0.506	3
B7	PET	10	(GT)11	6	91-113	0.09	0.5	0.534	0.493	3
F8	VIC	11	(AC)30	5	105-147	0.1	0.400	0.667	0.665	1
L5	VIC	6	(CA)13	2	89-92	0.03	0.5	0.508	0.371	3
ReMs*a*7										
L2	6-FAM	12	(TG)10	5	87- 95	0.07	0.857	0.738	0.667	1*
G2	6-FAM	13	(CA)15	8	113-135	0.1	0.7	0.774	0.739	3
G3	6-FAM	26	(TG)19	8	150-172	0.08	0.867	0.807	0.736	3
G4	NED	15	(AC)17	6	83-93	0.06	0.909	0.814	0.768	3
G5	NED	24	(AC)22	8	113-147	0.1	0.800	0.874	0.808	3
G7	PET	14	(CA)11	8	84-112	0.1	0.999	0.848	0.785	3
G8	VIC	9	(CA)14	4	80-92	0.04	0.818	0.749	0.649	2
G9	VIC	1	(CA)15	8	106-124	0.05	0.917	0.790	0.716	3
ReMs*a*8										
J1	6-FAM	5	(GT)14	7	74-98	0.06	0.938	0.806	0.748	3
J3	6-FAM	5	(TG)30	16	112-178	0.1	0.938	0.95	0.914	3
J5	VIC	9	(TG)18	10	107-160	0.07	0.875	0.827	0.782	1
J7	NED	21	(GT)12	3	91-97	0.05	0.188	0.28	0.248	3
J8	NED	15	(CA)21	5	104-120	0.07	0.625	0.613	0.554	3
J9	NED	1	(GT)43	12	126-159	0.1	0.999	0.885	0.841	3
J10	PET	12	(AC)15	5	91-104	0.08	0.625	0.732	0.656	3
J11	PET	2	(TG)18	8	116-141	0.09	0.813	0.796	0.742	3
ReMs*a*9										
K1	6-FAM	3	(CA)12	5	72-104	0.06	0.5	0.468	0.422	2
F2	6-FAM	13	(CA)31	9	108-148	0.09	0.636	0.797	0.734	3
K4	VIC	25	(TG)10	5	79-95	0.08	0.75	0.645	0.57	3
K6	VIC	14	(CA)20	8	100-126	0.06	0.875	0.835	0.787	3
K7	NED	7	(AC)12	8	79-101	0.06	0.688	0.813	0.759	3
K9	NED	3	(CA)25	10	106-134	0.09	0.75	0.887	0.844	1
K10	PET	19	(CA)14	5	89-103	0.08	0.563	0.724	0.654	2
K11	PET	17	(TG)17	5	111-127	0.08	0.5	0.732	0.656	2
ReMs*a*10										
L1	6-FAM	5	(GT)11	3	71-86	0.04	0.563	0.567	0.452	3
C2	6-FAM	1	(CA)13	7	99-131	0.08	0.867	0.749	0.683	2*
L7	NED	16	(CA)11	3	74-78	0.04	0.75	0.534	0.412	2
L8	NED	8	(TG)13	4	89-108	0.07	0.563	0.542	0.486	3
L9	NED	20	(GT)15	3	118-127	0.07	0.563	0.575	0.457	3
L10	PET	9	(AC)12	3	76-86	0.07	0.625	0.462	0.371	3
L11	PET	10	(GT)14	5	101-111	0.08	0.75	0.669	0.606	3
L12	PET	25	(TG)21	5	121-138	0.08	0.375	0.339	0.313	3
L4	VIC	12	(AG)13	2	76-86	0.05	0	0.138	0.124	3
L6	VIC	4	(CA)22	2	96-121	0.07	0.25	0.315	0.258	3
ReMs*a*11										
G1	6-FAM	17	(TG)15	13	82- 152	0,9	0,947	0,89	0.893	3
M6	NED	11	(AC)12	3	94- 98	0.09	0.4	0.628	0.539	3
M7	NED	18	(CA)22	8	114- 140	0.09	0.8	0.766	0.703	3
M8	PET	13	(TG)13	8	68- 98	0.07	0.133	0.798	0.746	3

Table 2. Continued

M9	PET	24	(AC)20	5	112- 120	0.08	0.8	0.752	0.678	3
M3	VIC	7	(CA)16	6	81- 93	0.07	0.667	0.68	0.601	3
A11	VIC	25	(CA)23 (GA)5	11	97- 135	0.06	0.75	0.913	0.874	3
I9	VIC	19	(TAGA)13	5	153- 169	0.06	0.5	0.623	0.551	3
ReM*sa*12										
K2	6-FAM	3	(GT)14	8	86-117	0.06	0.875	0.855	0.806	3
I2	6-FAM	20	(GT)18	12	121-161	0.1	0.938	0.923	0.885	3
C6	NED	8	(CA)15	4	97-103	0.04	0.313	0.421	0.375	3
C9	PET	7	(GT)16	5	109-123	0.1	0.4	0.687	0.603	3
D12	VIC	23	(AC)27	10	88-119	0.09	0.999	0.887	0.842	3
I8	VIC	3	(GT)24	7	126-142	0.09	0.938	0.754	0.697	3
ReM*sa*13										
I1	6-FAM	23	(TGG)3 (TG)9	3	96-102	0.05	0.188	0.373	0.327	3
C3	6-FAM	6	(TG)27	13	106-140	0.08	0.933	0.92	0.879	3
H4	NED	17	(CA)15	6	92-104	0.1	0.875	0.758	0.692	3
G6	PET	18	(AC)17	6	84-112	0.15	0.727	0.771	0.694	3
D10	PET	18	(CA)40	8	126-155	0.15	0.563	0.786	0.734	3
H8	VIC	19	(TG)16	3	108-116	0.05	0.875	0.677	0.582	3
D13	VIC	10	(AC)29	11	123-172	0.15	0.6	0.897	0.853	3
Alternative microsatellite markers										
A 6	-	12	(CA)27	8	107 - 133	-	0.214	0.881	0.832	3
A 9	-	2	(TG)31	8	132 - 152	-	0.667	0.793	0.74	3
B 1	-	2	(CA)16	9	79 - 115	-	0.733	0.816	0.762	3
B 5	-	8	(TCC)12	2	102 - 117	-	0.077	0.471	0.350	3
B 10	-	6	(GT)18	5	97 - 135	-	0.467	0.587	0.388	1
D 6	-	11	(GT)26	11	81 - 112	-	0.999	0.853	0.811	3
D 2	-	24	(CA)19	10	87 - 120	-	0.938	0.897	0.856	3
E 5	-	13	(CA)20	9	90 - 112	-	0.875	0.917	0.844	3
E 8	-	4	(GT)11	6	114 - 139	-	0.364	0.756	0.544	3
F 5	-	6	(GT)13	6	114 - 148	-	0.500	0.742	0.663	3
H 1	-	14	(GT)16	11	81 - 119	-	0.813	0.810	0.767	3
H 5	-	25	(AC)25	7	103 - 121	-	0.600	0.694	0.644	3
H 6	-	26	(GT)17	13	83 - 141	-	0.438	0.915	0.876	3
I 3	-	15	(GT)18	4	101 - 115	-	0.600	0.687	0.597	2
I 4	-	26	(CA)16	6	105 - 119	-	0.867	0.805	0.749	3
I 6	-	7	(CA)24	7	110 - 140	-	0.938	0.859	0.81	3
I 5	-	2	(GT)11	5	102 - 122	-	0.500	0.611	0.551	3
K 3	-	14	(TG)15	7	104 - 134	-	0.400	0.772	0.719	3
K 8	-	3	(CA)26	14	81 - 134	-	0.867	0.931	0.892	3
L 3	-	9	(GT)28	10	73 - 137	-	0.400	0.768	0.714	2

GremmProtocol (Lee-Montero *et al.*, 2013) evaluates the reliability of each microsatellite genotyping by quantification of genotyping errors or potential errors as: 1 (ambiguous genotypes in more than 30% of the samples), 2 (ambiguous genotypes in 30% or less of the samples) and 3 (unambiguous genotypes in 100% of the samples). * New score after validation samples genotyping

2.4.2. Multiplex PCRs validation and genotyping reliability revaluation

The correct inheritance and segregation of alleles of each locus between parents and offspring was confirmed after the validation samples were genotyped. The alleles of these samples were within the size range described for the 16 control samples. The microsatellite markers that showed null alleles (allelic frequency in parents: 0.25), detected through familial

segregation, were C11, E9, I1, K9, M3 and M8. After revaluation by the *GremmProtocol*, only the scores of the markers C2, H9, L2 and M2 changed. The marker C2 changed from 3 to 2 and the marker L2 from 2 to 1 due to an “intermediate allele” genotyping error found in both markers. The scores of H9 and M2 changed from 2 to 3 because any potential genotyping error was not found in the 62 validation samples.

2.5. Discussion

Microsatellite-based strategies have been successfully used to identify QTLs of economic interest in many aquaculture species (Wang *et al*., 2006; Massault *et al*., 2010; Sánchez-Molano *et al*., 2011; Norman *et al*., 2012). Their genome-wide distribution and high levels of allelic polymorphism (Chistiakov *et al*., 2006) make them greatly used tools in QTL search since, in this case, genotyping many individuals with a high number of loci is required. For this very reason, the use of these markers by multiplex PCR offers a desirable reduction of costs per sample (Navarro *et al*., 2008). The 13 new multiplex PCRs developed in this study, including a total of 106 microsatellites, constitute the first set of multiplex PCRs to identify QTL in gilthead seabream. Several studies have used microsatellites markers for QTL detection in this species (Massault *et al*., 2010; Boulton *et al*., 2011; Loukovitis *et al*., 2011, 2012, 2103), but none of them have used a set of multiplex PCRs. Two of these studies used a multiplex PCR with 9 microsatellites, but only to determine the family relationship (Boulton *et al*., 2011; Loukovitis *et al*., 2011). For QTL searching, these authors used additional markers by single PCR reactions.

Other multiplex PCRs have been developed in gilthead seabream for parentage assignment and/or populations genetic studies (Launey *et al*., 2003; Brown *et al*., 2005; Navarro *et al*., 2008; Porta *et al*., 2010; Borrell *et al*., 2011; Vogiatzi *et al*., 2011; Lee-Montero *et al*., 2013), but none have included as many microsatellites as this study. Actually, a genetic linkage map with a high level of saturation of molecular markers is needed for an effective QTL detection (Lynch and Walsh, 1998). The length of the genetic linkage map of gilthead seabream is 1241.9 cM and it presents 26 LG (Franch *et al*., 2006). Apart from the 22 microsatellites that conform the SMsa1 and SMsa2 (Lee-Montero *et al*., 2013), the 13 multiplex PCRs developed in this study are the only ones whose microsatellites are all located

on the genetic map of this species. In fact, these 13 multiplex reactions cover 100% of LG of the genetic map of this species, thus making this a powerful and low-cost tool to identify QTL. Moreover, the 13 multiplex PCRs work under the same conditions, thereby making it possible for several multiplex reactions to be executed in a unique PCR run or exchange markers between assays. Additionally, the 20 alternative microsatellite markers not included in any multiplex PCR offer geneticists the possibility to include them later in further screening, given that their corresponding primers have also been redesigned to be amplified under the same PCR conditions. A further advantage of all the markers included in this study is that the amplicons are small (< 200 bp). These similar sizes should avoid differential amplification of size variants due to the competitive nature of PCR by which short-length alleles often amplify more efficiently than longer ones (Dakin and Avise, 2004), especially when DNA quality is low (Pompanon *et al*., 2005).

On the other hand, QTL searching is more efficient with highly polymorphic markers (Lynch and Walsh, 1998). Most of the multiplex reactions proposed in this study showed high mean values of Ho and He, similar to other multiplex PCRs in gilthead seabream (Navarro *et al*., 2008; Borrell *et al*., 2011; Lee-Montero *et al*., 2013). Likewise, 74.5% of the markers that make up these multiplex reactions are highly informative according to their PIC values. However, in ReM*sa*3 and ReM*sa*10, the mean PIC values were somewhat lower. Nevertheless, the objective of this study was to create a set of multiplex PCRs to identify QTL, so the maximum possible number of markers to carry out a comprehensive sweep of the map was included, even though the mean variability values fell. Higher genetic variability values are needed to determine pedigree, but highly efficient multiplexes to this end already exist for this species (Navarro *et al*., 2008; Borrell *et al*., 2011; Lee-Montero *et al*., 2013). In fact, the genetic variability values measured in mean allele numbers found in the microsatellite markers of this study are very similar to others used in QTL search (8.1 for the

22 microsatellite markers used for gilthead seabream in Loukovitis *et al*., 2011) or even higher (4.57 for European sea bass, *Dicentrarchus labrax* L. in Massault *et al*., 2010).

When a new multiplex PCR is developed, its success requires evaluation and validation (Markoulatos *et al*., 2002; Navarro *et al*., 2008). Hence, the correct inheritance and segregation of alleles of each locus of the multiplex PCRs developed in this study was confirmed after the genotyping of the 62 validation samples. The multiplex PCR approach minimizes the genotyping errors since the laboratory work is greatly simplified and automated (Navarro *et al*., 2008). However, genotyping errors due to the inherent characteristics of each microsatellite may complicate sample analysis, which increase costs when a high number of individuals are genotyped. The genotyping reliability of the 138 microsatellites used in this study was evaluated by the *GremmProtocol* proposed by Lee-Montero *et al*. (2013). This identification is crucial for correct genotype scoring. In this study, a new genotyping reliability evaluation with this protocol of 106 markers that comprise the multiplex PCRs used was carried out. Only four markers presented different values than in the first evaluation with the 16 control samples, confirming the validity of the model. It is known that a possible drawback of microsatellite markers genotyping is the presence of null alleles, which leads to false exclusions when heterozygotes are considered as homozygotes (Borrell *et al*., 2004; Navarro *et al*., 2008). Although null alleles are frequently found in interspecific microsatellites, they have been found in studies with specific markers in gilthead seabream (Lee-Montero *et al*., 2013). In this study, the 5.66 % of microsatellites showed null alleles. However, this does not constitute a trouble when determining QTL because null alleles would be easily identifiable since pedigree data are available. In fact, once they have been identified, they could be recoded manually and used as a further allele (Navarro *et al*., 2008).

Conclusion

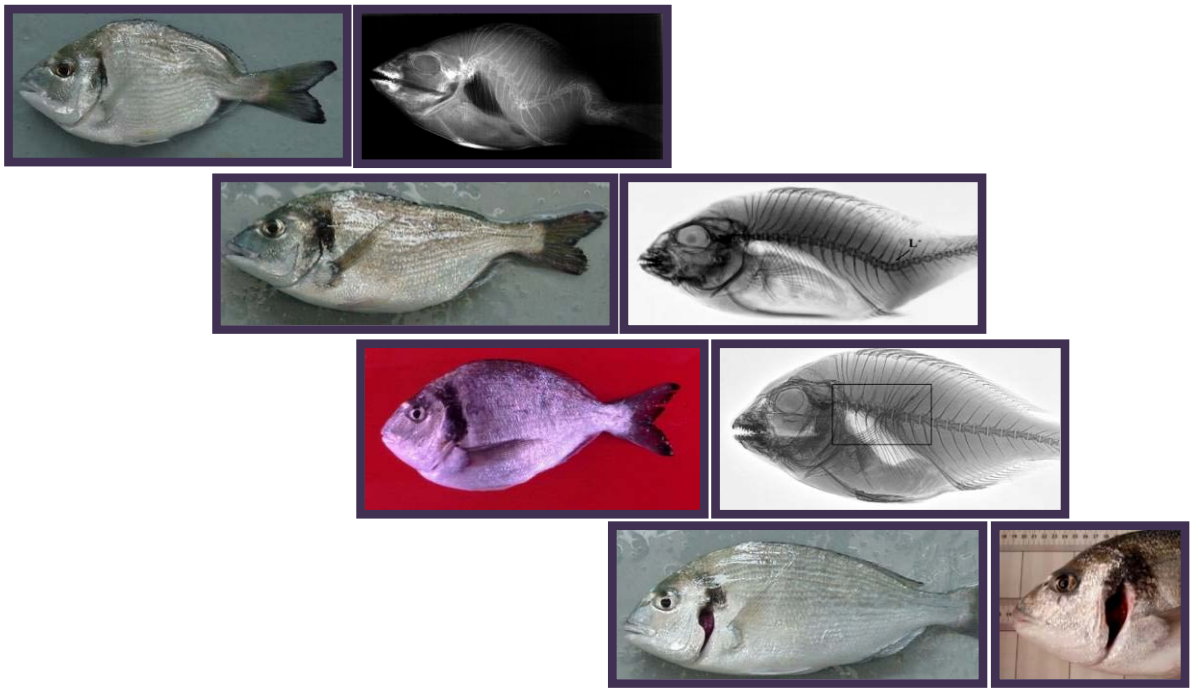
This study provides a notably useful tool for QTL identification in gilthead seabream. 106 microsatellite markers evaluated for genotyping reliability have been combined in 13 new

multiplex PCR reactions that provide a good level of saturation for genetic linkage studies in this species, as well as, minimize the cost per reaction and genotyping errors.

Table 3. Number of loci (N) and genetic variability for each multiplex PCR (ReM*sa*) developed in this study on gilthead seabream: Mean Observed Heterozygosity, Mean Expected Heterozygosity, Mean Polymorphism Information Content, Combined non-exclusion probabilities (CnEP) and Mean number of alleles per locus

Multiplex	**N**	**Mean Ho**	**Mean He**	**Mean PIC**	**CnEP (PP)**	**CnEP (SP)**	**Mean NA**
ReM*sa*1	10	0.671	0.603	0.671	$8.90\cdot10^{-07}$	$2.84\cdot10^{-04}$	6.910
ReM*sa*2	8	0.710	0.734	0.681	$1.50\cdot10^{-06}$	$4.11\cdot10^{-04}$	8.630
ReM*sa*3	8	0.552	0.632	0.570	$3.49\cdot10^{-04}$	$9.09\cdot10^{-03}$	5.500
ReM*sa*4	8	0.676	0.688	0.637	$1.45\cdot10^{-05}$	$1.50\cdot10^{-03}$	7.750
ReM*sa*5	9	0.688	0.740	0.679	$9.40\cdot10^{-07}$	$2.87\cdot10^{-04}$	8
ReM*sa*6	8	0.726	0.656	0.726	$1.38\cdot10^{-05}$	$1.49\cdot10^{-03}$	7.380
ReM*sa*7	8	0.859	0.799	0.734	$3.78\cdot10^{-06}$	$5.99\cdot10^{-04}$	6.780
ReM*sa*8	8	0.750	0.736	0.686	$6.17\cdot10^{-06}$	$9.00\cdot10^{-04}$	8.250
ReM*sa*9	8	0.658	0.738	0.678	$3.55\cdot10^{-05}$	$2.41\cdot10^{-03}$	6.880
ReM*sa*10	10	0.531	0.489	0.416	$5.34\cdot10^{-03}$	$4.66\cdot10^{-02}$	3.700
ReM*sa*11	8	0.625	0.757	0.698	$1.13\cdot10^{-05}$	$1.22\cdot10^{-03}$	7.380
ReM*sa*12	6	0.744	0.755	0.701	$1.07\cdot10^{-04}$	$4.60\cdot10^{-03}$	7.670
ReM*sa*13	7	0.680	0.740	0.680	$5.99\cdot10^{-05}$	$3.22\cdot10^{-03}$	7.140

PP: parent pair option, SP: second parent option



3. Segregation Analysis of Skeletal Deformities in the Gilthead Seabream *Sparus aurata* L.); Lack of Operculum, Lordosis, Vertebral Fusion and LSK

Negrín-Báez *et al*. (2014b). Journal of Animal Science. In revision

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Oral comunication. XVII Reunión de Mejora Genética Animal. 5-6 junio de 2014. Bellaterra. Barcelona

3.1. Abstract

Morphological abnormalities in farmed gilthead seabream (*Sparus aurata* L.) are a major problem and it entails significant economic losses. In this study, three large scale experiments were carried out under different conditions of spawning, offspring handling and breeders phenotype in order to analyze the segregation of four types of deformities in this species. Lack of operculum, lordosis and vertebral fusion are three of the most important skeletal deformities and LSK is a consecutive repetition of lordosis/scoliosis/kyphosis. In Experiment 1 (mass spawning and fingerling sorting), 900 fish were analyzed at 509 days post-hatching: 846 fish on-grown in a farm and 54 LSK fish that had been selected during fingerling sorting and reared separately. Of the 89 families represented, a statistically significant association between five of them (formed by six breeders) and LSK fish was found. In Experiment 2 (mass spawning and no fingerling sorting), 810 fish were analyzed at two ages. Significant relationships between two of the breeders and two of the families with the lack of operculum prevalence of their descendants were found at 539 days post-hatching but not at 179 days post-hatching. This suggests that environmental factors effect to lack of operculum is higher at early age. Prevalence of column deformities was low and no association with family structure was observed. Family relationships were determined by microsatellites multiplex PCR in both experiments. In Experiment 3 (designed mating), sires suffering from lordosis or lack of operculum or vertebral fusion deformities were mated with non-deformed dams and a mass-spawning control mating was considered. After analyzing 11503 offspring at 159 days post-hatching, the results showed a significant relationship between the prevalence of each deformity and the mating of breeders suffering from the same deformity. However, a significant prevalence of lack of operculum was also observed in lordotic mating. This suggests that lordosis and vertebral fusion deformity are related to

family structure, while lack of operculum is related to environmental factors. The results of the present study report that the analyzed deformities prevalence has a genetic origin although it is also influenced by environmental factors. Producers should consider both factors to improve significantly their fish morphological quality and to minimize the incidence of deformities in farmed fish.

3.2. Introduction

The presence of morphological abnormalities in farmed gilthead seabream (*Sparus aurata* L.) is currently a major problem in aquaculture as it entails significant economic losses (Afonso and Roo, 2007; reviewed by Bardon *et al.*, 2009). Skeletal deformities are the most relevant deformities and they include head and vertebral column anomalies. Lack of operculum in head, and lordosis, scoliosis, kyphosis and vertebral fusion in column, are the most frequent skeletal anomalies in this species (Galeotti *et al.*, 2000; Beraldo and Canavese, 2011; reviewed by Boglione *et al.*, 2013). They affect the fish appearance and lead to physiological alterations that result in a devaluation of their commercial traits (Gjerde *et al.*, 2005; Karahan *et al.*, 2013).

High number of studies has determined that environmental factors are linked to fish deformities (reviewed by Bardon *et al.*, 2009), but only a few of them have contributed to their genetic determination. In gilthead seabream, a significant family association for a triple column deformity (LSK complex, lordosis-scoliosis-kyphosis) was found by Afonso *et al.* (2000) and a high heritability for presence/absence of any kind of deformities was estimated by Astorga *et al*. (2004). Lee-Montero *et al*. (2014) estimated medium value of heritabilities for deformity traits in fish reared in four Spanish regions (PROGENSA®). These results suggest that skeletal deformities variability in a particular population, besides being influenced by the environment, can also be explained by genetic origin.

The main objective of the present study was to analyze in gilthead seabream the prevalence of four skeletal deformities (lack of operculum, lordosis, vertebral fusion and LSK complex) associated with the family structure and considering different breeding conditions, phenotype of breeders and offspring handling; in order to study how genetic predisposition and management of breeders can determine the emergence of these deformities in offspring.

3.3. Material and Methods

Three experiments to assess the prevalence of deformity in the offspring from different broodstocks were carried out at the facilities of the Marine Science and Technology Park of the University of Las Palmas de Gran Canaria (PCTM-ULPGC, Gran Canaria, Spain), which belongs to the Foundation of Science and Technology Park of ULPGC (FCPCT-ULPGC).

3.3.1. Experiment 1 (mass spawning with sorting)

Breeders and mating structure.

An egg batch was collected by using mass spawning from an industrial hatchery broodstock. This broodstock was composed of 66 non-deformed breeders and the sex ratio of which was 1♀: 2♂.

Offspring and rearing conditions.

Eggs and larvae were reared at PCTM-ULPGC and under the conditions described by Roo *et al.* (2009). Fingerlings were sorted by presence *vs* absence of deformity at 111 days post-hatching (1.9 ± 0.02 g) (mean ± standard error), as performed by companies. Deformed fish were culled with the exception of LSK (lordosis/scoliosis/kyphosis complex) fish that were selected. The percentage of LSK found in the whole batch of fingerlings was 0.22%. All LSK fish were reared at PCTM-ULPGC separately in a 1000 L fiberglass tank. Water temperature ranged from 19.3 ± 0.1 °C in March to 25.0 ± 0.1 °C in September, and values for dissolved oxygen and water flow were 6.0 ± 0.1 ppm and 21 $l \cdot min^{-1}$, respectively. Commercial feed was provided through self-feeders. With respect to fish considered as normal or without abnormalities, they were taken to the facilities of Playa de Vargas 2001 S.L. Company (PLV2001, Gran Canaria, Spain) at 130 days post-hatching (4.8 ± 0.1 g). The fish at PLV2001 were reared under industrial conditions in a cage and fed with commercial

fish feed. Dissolved oxygen in the water had an average value of 7.4 ppm. The on-growing period lasted up to 509 days post-hatching when fish were 419.7 ± 3.1 g.

Visual assessment.

At the end of the experiment, a sample of 846 fish from PLV2001 was slaughtered and visually analyzed to determine the presence or absence of deformities according to AquaExcel-ATOL (AquaExcel Project, 2013; ATOL: 0000087). Vertebral deformities were assessed, after being filleted, by direct observation of the axial skeleton.

Parental assignment.

Family relationships between breeders and offspring (846 fish from PLV2001 and 54 LSK fish from PCTM-ULPGC) were determined by the exclusion method using the software VITASSING (v8.2.1) (Vandeputte *et al.*, 2006), after DNA analysis and genetic characterization by using the multiplex PCR SMsa1 (*SuperMultiplex Sparus aurata*), as described by Lee- Montero *et al.* (2013).

3.3.2. Experiment 2 (mass spawning without sorting)

Breeders and mating structure.

Egg batches were collected on two consecutive days from a mass spawning of an industrial broodstock of PCTM-ULPGC within the context of the PROGENSA® breeding program (Afonso *et al.*, 2012). This broodstock was composed of 59 non-deformed breeders, the sex ratio of which was 1♀: 1.81♂.

Offspring and rearing conditions.

Eggs and larvae were cultured at the PCTM-ULPGC facilities as described in Experiment 1. At day 179 post-hatching (17.2 ± 0.2 g), fingerlings were individually tagged with Passive Integrated Transponder (PIT; Trovan Daimler-Benz) by following the tagging protocol described by Navarro *et al.* (2006) and transported to a cage of CANEXMAR S.L. company (Gran Canaria, Spain). They were reared under intensive conditions and fed with commercial feed. Dissolved oxygen in the water had an average value of 7.4 ppm and water

temperature ranged from 20.2°C to 24.2°C. No sorting or culling processes were performed during larval rearing or on-growing periods, which lasted up to 689 days post-hatching, when fish were 524.4 ± 12.6 g.

Visual assessment.

When fish were tagged (179 days post-hatching), they were also observed in order to determine the presence or absence of deformities (initial analysis) (ATOL: 0000087). At the end of the experiment (689 days post-hatching), a sample of 810 fish were slaughtered and visually analyzed (final analysis) to determine the presence or absence of deformities (ATOL: 0000087). Vertebral deformities were directly assessed after being fish filleted like in Experiment 1.

Parental assignment.

Family relationships between breeders and offspring (810 fish) were determined as described in Experiment 1.

3.3.3. Experiment 3 (designed mating)

Breeders and mating structure.

Different directed matings were established using gilthead seabream breeders from PCTM-ULPGC facilities, more concretely, 15 deformed sires and 10 normal (N, non-deformed) dams. Deformed fish were classified into three groups: Lack of operculum (O), Lordosis (L) and Vertebral Fusion (VF). Three matings (replica) by each deformity were constituted: NxO, NxL and NxVF. The sex ratio in each tank was one normal dam with two deformed sires ($1♀_N$: $2♂_D$). Additionally, a broodstock of non-deformed breeders ($1♀_N$: $2♂_N$) was used to conform a Control mating (**C**) by mass spawning.

Offspring and rearing conditions.

Eggs from each mating were separately cultured in PCTM-ULPGC facilities as described in Experiment 1. Fingerlings from each mating were separately reared in 1000 L fiberglass tanks until the end of the experiment (two tanks *per* type of mating). Rearing conditions were as

follow: commercial feed was provided by automatic feeders; water flow was 0.5 $l \cdot min^{-1}$, dissolved oxygen concentration was 5.9 ± 0.1 ppm and water temperature ranged from 19.3 ± 0.1ºC at the beginning of the experiment to 23 ± 0.1ºC at the end. The experiment ended at 129 days post-hatching (9.6 ± 0.1 g).

Visual assessment.

At the end of the experiment, all fingerlings from all mating types, including mass spawning, (11503 fish) were visually analyzed to determine the presence or absence of deformities (ATOL: 0000087).

3.3.4. Statistical analysis

The identified deformities were: lordosis (L), vertebral fusion (VF), lack of operculum (O) and lordosis/scoliosis/kyphosis complex (LSK). Fish that did not show any type of these deformities were considered as normal (N). The prevalence rate of deformities was calculated as a percentage of deformed descendants with respect to the total of analyzed fish in each group (breeder, family, mating or experiment).

The association between any factor (breeder, family, mating or experiment) and deformity, was analyzed by a log linear model by using the statistical software SPSS (PASW Statistics v18). Log linear model gives the significance of any deformity factor (its prevalence [*i*]) against any biological or functional factor (depending of data [*j*]), organized under a two-way contingency table, through the normalized values or Z values. Normalized Z values > +1.96 or <-1.96 indicate an excess or defect statistical significance of deformity, respectively, in any family, breeder, mating or experiment.

$$ln f_{ij} = \mu + \alpha_i + \beta_j + \alpha\beta_{ij}$$

Where, $ln f_{ij}$ is the expected frequency of each deformity (i) in each family, breeder, mating or experiment (j) considered (ij); μ is the average value of expected frequencies logarithms, α_i is

the effect of the deformity factor (i), β_j is the effect of the family, breeder, mating or experiment (j) and $\alpha\beta_{ij}$ is the effect due to these factors interaction.

In experiments 1 and 2 was analyzed the relationship between each deformity and all families, sires and dams. In experiment 3, was analyzed the relationship between each deformity and all mating, families and tanks.

3.4. Results

The total deformities prevalence of offspring of the broodstock in the three experiments is showed in Table 4.

Table 4. Deformities prevalence (%) in total offpring in each experiment

	Operculum	Lordosis	Vertebral fusion	LSK	Normal	N
Experiment 1	0.44	5.67	0.44	6	87.44	900
Experiment 2	5.80	1.73	0.12	0	92.35	810
Experiment 3	4.84	0.64	0.63	0.03	93.85	10650

N: Numbers of descendants from each experiment. Experiment 1: Mass spawning with sorting. Experiment 2: mass spawning without sorting. Experiment 3: Only designed mating with deformed breeders.

3.4.1. Experiment 1

Of the total of LSK fish that were selected, only 54 survived at the end of the experiment (75% of mortality). A 100% success was obtained in the parental assignment, i.e. whole offspring (900 individuals) were assigned to 28 breeders (17 dams and 11 sires). A total number of 89 full-sibling families were represented, and only six of them included the total of LSK individuals (54). These families (named msF1-msF6) were composed of six breeders: four dams and two sires (named ms♀1-ms♀4 and ms♂1-ms♂2). Deformities prevalence (with its Z values) in the descendants of these dams and sires compared with the other breeders (mean value of the prevalence), and with respect to each sire, dam and family, are shown in Table 5. Both sires, the three dams and five families showed a statistically significant relationship with deformity due to their high prevalence of LSK fish ($Z_{family,\ LSK} > +2$; $P<0.05$). These families had from 28.57 to 66.67 % of LSK deformed descendants. It is remarkable that ms♂1 and ms♂2 were responsible for 75% of vertebral fusion deformed fish, while ms♀2 was responsible for 51.85% of the LSK deformed fish and 25% of the fish with lack of operculum. None of the dams showed any descendant with VF deformity.

Table 5. Deformities prevalence (%) in descendants of breeders responsible of LSK deformed fish (lordosis/scoliosis/kyphosis complex) with respect to each sire, dam and family of the Experiment 1

	Operculum	Lordosis	Vertebral fusion	LSK	Normal	N
Sires						
ms♂1	0 $_{-0.95}$	4.52 $_{-1.40}$	0.65 $_{-0.24}$	**22.58** $_{\mathbf{3.62}}$	72.26$_{-1.03}$	155
ms♂2	0.94$_{-1.55}$	4.72 $_{-0.52}$	1.89 $_{-0.80}$	**17.92** $_{\mathbf{4.72}}$	74.53 $_{-1.85}$	106
Mean ms♂^{R}	0.34	6.77	0.10	0	92.79	639
Dams						
ms♀1	0 $_{-0.93}$	3.15 $_{-0.90}$	0 $_{-0,59}$	**11.81** $_{\mathbf{3.75}}$	85.04 $_{-1.34}$	127
ms♀2	1.20 $_{-0.14}$	3.61 $_{-1.04}$	0 $_{-1,33}$	**33.73** $_{\mathbf{5.37}}$	**61.45** $_{\mathbf{-2.86}}$	83
ms♀3	0 $_{-0.37}$	7.50 $_{-0.34}$	0 $_{0,11}$	5 $_{1.06}$	87.50 $_{-0.47}$	40
ms♀4	0 $_{-0.19}$	0 $_{-0.16}$	0 $_{-1,47}$	**27.27** $_{\mathbf{3.83}}$	**72.73** $_{\mathbf{-2.01}}$	33
Mean ms♀^{R}	0.21	13.29	0.43	0	86.07	617
Families						
msF1 (♀1♂1)	0 $_{-0.62}$	0$_{-1.00}$	0$_{-0.62}$	**48.15** $_{\mathbf{4.42}}$	**51.85** $_{\mathbf{-2.18}}$	27
msF2 (♀1♂2)	0 $_{-0.18}$	0$_{-0.55}$	0$_{-0.18}$	28.57 $_{1.78}$	71.43 $_{-0.87}$	7
msF3 (♀2♂1)	0 $_{-0.82}$	3.33$_{-0.41}$	0$_{-0.82}$	**66.67** $_{\mathbf{5.43}}$	**30** $_{\mathbf{-3.38}}$	30
msF4 (♀2♂2)	0 $_{-0.63}$	6.25$_{0.12}$	0$_{-0.63}$	**50** $_{\mathbf{3.73}}$	**43.75** $_{\mathbf{-2.36}}$	16
msF5 (♀3♂1)	0 $_{0.04}$	0$_{-0.33}$	0 $_{0.04}$	**66.67** $_{\mathbf{2.10}}$	33.33 $_{-1.85}$	3
msF6 (♀4♂2)	0 $_{-0.45}$	0$_{-0.83}$	0$_{-0.45}$	**56.25** $_{\mathbf{4.01}}$	**43.75** $_{\mathbf{-2.28}}$	16
Mean msF^{R}	0.95	6.75	0.64	0	91.66	801

Z values subscript. Significant associations when Z≥+1.96; Z≤-1.96 (Bold characters). N: Number of descendants. ^{R}: Mean of prevalence from the rest of males, females and families

3.4.2. Experiment 2

100% success was also obtained in the parental assignment, i.e. whole offspring (810 individuals) were assigned to 45 breeders (19 dams and 26 sires) and 66 full-sibling families were formed. In the initial analysis at 179 days post-hatching, 7% of offspring showed lack of operculum, and neither lordosis nor vertebral fusion deformities were observed. Deformity prevalence was independent of family or breeder ($-1.96<Z_{breeder/family,deformity}<+1.96$; $P>0.05$). At 689 days, at the final analysis, the total prevalence of deformities was 7.65%. Only the operculum deformity prevalence showed a significant statistically association ($Z>+2$, $P<0.05$) with sires, dams and the families formed by these breeders. 48.94% of individuals with lack of operculum were descendant of one sire (named ms♂1), which represents a significant

prevalence. Only two families, the formed by mw♂1 and two dams (named mw♀1 and mw♀3), showed a significant prevalence of operculum deformity, as they represented 12.77 and 21.28% of all the individuals, respectively. The deformities prevalence in offspring from mw♂1 at final analysis is shown in Table 6.

Table 6. Deformities prevalence (%) in offspring from mw♂1 of the Experiment 2

	Operculum	**Lordosis**	**Vertebral fusion**	**Normal**	**N**
Sires					
mw♂1	**14.84** $_{\mathbf{3.24}}$	0 $_{-1.59}$	0 $_{-1.27}$	85.16 $_{-0.37}$	155
Mean mw♂^{R}	6.15	3.13	0.02	90.70	655
Dams					
mw♀1	**17.54** $_{\mathbf{2.01}}$	1.75 $_{-0.50}$	0 $_{-0.89}$	80.70 $_{-0.62}$	57
mw♀2	3.66 $_{0.47}$	0 $_{-0.95}$	0 $_{-0.48}$	96.34 $_{0.97}$	82
mw♀3	4.62 $_{0.59}$	1.54 $_{-0.52}$	0.31 $_{-1.56}$	93.54 $_{1.50}$	325
mw♀4	4.69 $_{0.16}$	1.56 $_{-0.21}$	0 $_{-0.69}$	93.75 $_{0.73}$	64
mw♀5	2.08 $_{-0.66}$	2.08 $_{0.18}$	0 $_{-0.42}$	95.83 $_{0.90}$	48
mw♀6	4.59 $_{0.17}$	2.75 $_{0.44}$	0 $_{-1.13}$	92.66 $_{0.52}$	109
Mean mw♀^{R}	10.97	4.14	0	84.89	125
Families					
mwF1(♀1♂1)	**46.15** $_{\mathbf{2.57}}$	0 $_{-0.66}$	0 $_{-0.51}$	53.85 $_{-1.41}$	13
mwF2(♀2♂1)	20 $_{0.69}$	0 $_{-0.22}$	0 $_{-0.06}$	80 $_{-0.41}$	5
mwF3(♀3♂1)	**11.63** $_{\mathbf{2.34}}$	0 $_{-1.29}$	0 $_{-1.14}$	88.37 $_{0.10}$	86
mwF4(♀4♂1)	7.69 $_{0.38}$	0 $_{-0.44}$	0 $_{-0.29}$	92.31 $_{0.36}$	13
mwF5(♀5♂1)	12.50 $_{0.54}$	0 $_{-0.33}$	0 $_{-0.18}$	87.50 $_{-0.03}$	8
mwF6(♀6♂1)	13.33 $_{1.51}$	0 $_{-0.86}$	0 $_{-0.71}$	86.67 $_{0.06}$	30
Mean mwF^{R}	6.23	2.40	0.01	91.35	655

Z values subscript. Significant associations when $Z \geq +1.96$; $Z \leq -1.96$ (Bold characters). N: Number of descendants. ^{R}: Mean of prevalence from the rest of males, females and families

3.4.3. Experiment 3

After three replicas of each type of mating, the following were viable: the three NxO, two NxL and only one NxVF. High mortality was observed in an NxFV and an NxL matings, so offspring from these matings could not be analyzed. From 11503 analyzed fish, 6.15% showed any of the analyzed deformities. The prevalence (with its Z values) of each type of deformity in offspring from different matings is shown in Table 7. As it can be observed, the

type of deformity was strongly associated to the type of mating: the NxO mating showed an excess of operculum deformity, the NxL mating showed an excess of operculum and lordosis deformities, the NxVF mating showed an excess of vertebral fusion deformity and the control mating showed an excess of normal fish ($Z_{mating,\ deformity} > +5$; $P<0.01$). The family effect (half-sibling), which only could be analyzed in two replicas of NxO mating, was statistically significant. One family showed an excess of operculum deformed fish (9.98%) and a defect of normal fish (89.54%), contrary to what was observed in the other family (0.98% and 99.69%, respectively) ($Z_{family,deformity} > +3.6$; $P<0.01$). In fact, when matings were analyzed with respect to the family, only one NxO family was responsible for the association between operculum deformity and NxO mating. However, no tank effect on prevalence of deformities was observed in mating or replica (during the rearing period) ($P>0.05$).

Table 7. Deformities prevalence (%) in offpring with respect to different matings of the Experiment 3

	Types of deformities					
Mating	**Operculum**	**Lordosis**	**Vertebral fusion**	**LSK**	**Normal**	**N**
NxO	**4.47** $_{\mathbf{5.61}}$	**0.18** $_{\mathbf{-2.48}}$	**0.1** $_{\mathbf{-3.60}}$	0.03 $_{0.12}$	95.22 $_{0.36}$	6803
NxL	**11.63** $_{\mathbf{4.85}}$	**2.47** $_{\mathbf{4.11}}$	**0.22** $_{\mathbf{-3.66}}$	0.05 $_{-0.24}$	**85.63** $_{\mathbf{-5.07}}$	1823
NxVF	**1.78** $_{\mathbf{-3.51}}$	0.69 $_{-0.52}$	**2.52** $_{\mathbf{5.51}}$	0.05 $_{-0.15}$	94.96 $_{-1.33}$	2024
Control	**0.59** $_{\mathbf{-6.95}}$	0.35 $_{-1.11}$	1.17 $_{1.75}$	0 $_{0.27}$	**97.89** $_{\mathbf{6.03}}$	853

Z values subscript. Significant associations when $Z \geq +1.96$; $Z \leq -1.96$ (Bold characters). N: Numbers of descendants from each mating. NxO= Operculum mating. NxL = Lordosis mating. NxVF= Vertebral Fusion mating. Control= Control mating

3.5. Discussion

Growth and deformities are the most economically important trait for the industrial production of gilthead seabream (Georgakopoulou *et al*., 2010). The most relevant deformities are those affecting opercular complex, neurocranium and vertebral column (Afonso and Roo, 2007; Izquierdo *et al*., 2010; reviewed by Boglione *et al*., 2013; Prestinicola *et al*., 2013). There are numerous studies on environmental effects on fish deformities in both, freshwater and marine aquaculture. Many of them have demonstrated that alterations on biotic and abiotic, physiological, xenobiotic, nutritional and rearing factors can be responsible for the development of fish deformities at an early stage (Boglione *et al*., 2001; reviewed by Bardon *et al*., 2009; Boglione and Costa, 2011; Prestinicola *et al*., 2013) or during their on-growing period (Lee-Montero *et al*., 2014). Additionally, genetic factors are also possibly responsible for the prevalence of different skeletal deformities in different species: Atlantic salmon (*Salmo salar*, Gjerde *et al*., 2005), rainbow trout (*Oncorhynchus mykiss*, Gislason *et al*., 2010; Kause *et al*., 2005), Atlantic cod (*Gadus morhua*, Kolstad *et al*., 2006), European seabass (*Dicentrarchus labrax*, reviewed by Bardon *et al.*, 2009, Karahan *et al*., 2013) and gilthead seabream (Afonso *et al*., 2000; Astorga *et al*., 2004, Lee-Montero *et al*., 2014). In this study, it has been conducted, for the first time in gilthead sebream, a four skeletal deformities segregation analysis, in order to determine their association with the family structure and its effect on prevalence of these deformities in offspring under industrial and experimental scale.

One of the reason by which deformities entail economic losses is the fact that companies have to perform manual sorting in order to select and eliminate deformed fingerlings at the end of the hatchery phase (Boglione and Costa, 2011). In Experiment 1, a commercial batch of 846 fish was analyzed to determine the prevalence of three of the most

frequent deformities in gilthead seabream production (lack of operculum, lordosis and vertebral fusion) at commercial size. Fish rearing was carried out under industrial conditions, so fish were sorted before being sold to an on-growing company. The sorting process was developed by flotation to exclude fish showing non-inflated swim bladder or severe skeletal anomalies (reviewed by Boglione *et al*., 2013). The only not excluded fish were LSK fish, which were selected and reared separately. LSK complex was described for the first time in gilthead seabream as a triple column deformity consisting of a consecutive repetition of lordosis/scoliosis/kyphosis from head to tail (Afonso *et al*., 2000). These authors designed a cross-breeding scheme which included separately rearing of 31 full-families and found a significant statistical interaction between the prevalence of this deformity and a family regardless of replicas. In their study, LSK fish represented 0.2% of all offspring; similarly to what was obtained in the present study: 0.22% of all the fish from the commercial batch. In this study, a statistically significant family correlation was also observed in LSK fish. Furthermore, a significant association between presence of this deformity and the descendants of 2 sires and 3 dams of the spawning-contributing breeders was observed. Five of the six families formed by these breeders presented a significant relationship with the high prevalence of this deformity and with the low prevalence of normal fish. In this study, moreover, the offspring was obtained by mass spawning and fingerlings were reared under de same conditions. From a genetic point of view, it has the advantage that common environmental sources are reduced (Herbinger *et al*., 1999). These results support the hypothesis of a genetic origin for LSK complex previously proposed by Afonso *et al*. (2000). Breeders forming these families were responsible for the 11.22% of lordotic fish and for the 75% of vertebral fusion fish (referred to 100% of analyzed fish). These data suggest a possible relationship between these deformities. However, no statistically significant relationship was observed between these deformities and any of the families or breeders, similarly to what was observed in lack of operculum. As expected from the fact that fish had

been previously sorted (during their fingerling stage), which makes it difficult to determine the genetic origin of any commercial trait. In this sense, Castro *et al*. (2008) also found no-different-from-zero heritability for lack of operculum and lordosis (0.02 ± 0.02 and 0.03 ± 0.02, respectively) in sorting gilthead seabream.

In Experiment 2, a batch of 810 gilthead seabreams were analyzed and the 4 deformities were evaluated, but no sorting or culling process were conducted during the fish rearing. At 179 days post-hatching (initial analysis), no deformity was detected with the exception of the lack of operculum. Deformities prevalence was lower (7%) than what has been observed in other studies on other sparids at this size (Roo *et al*., 2010; Boglione and Costa, 2011). This could be due to the fact that vertebral deformities are difficult to be detected at small sizes (reviewed by Bardon *et al*., 2009; Lee- Montero *et al*., 2014). The initial analysis of this experiment showed no relationship between operculum deformity prevalence and the any of the families or breeders. Contrastly, at 539 days, a significant relationship between two of the breeders (mw♂1 and mw♀1) and two of the families (mwF1 and mwF3) and the operculum deformity prevalence of their descendants was observed. Additionally, within the other families including this male, the operculum prevalence was much higher than the average of the experiment, although it was non-significant. Lee-Montero *et al*. (2014) also indicated a higher genetic determination for this deformity at commercial size (300-800 g) than at small size (15 g), as the estimation of heritability, being negligible at the initial point, increased until medium values (0.11) at commercial size. Additionally, these authors monitored individually tagged gilthead seabreams and demonstrated that some fish showing operculum deformity at small size recovered during their development. Thus, confirming the hypothesis that damaged operculum recovers by partial regeneration of their deformed elements (De Wolf *et al.*, 2004; Verhaegen *et al.*, 2007; Beraldo and Canavese, 2011). In fact, in this experiment, this deformity prevalence at final size is lower than at initial size. All these results suggest that the prevalence of lack of

operculum in small size fish is mainly influenced by the environment, whereas its genetic determination is more significant at larger sizes. In fact, the appearance of this deformity at small size has been widely related with culture or environmental factors (Koumoundouros *et al*., 1997; Beraldo *et al*., 2003; Roo *et al*., 2005; reviewed by Boglione *et al*., 2013). With respect to the other deformities, the prevalence was independent of family or breeder. So, these deformities could be caused by environmental factors. Accordingly, many studies have demonstrated the importance of environmental and physiological factors on skeletal deformities in fish (Boglione and Costa, 2011), which contrasts with other results in gilthead seabream, such as the following: Lee-Montero *et al*. (2014) estimated a heritability of 0.41 for any vertebral deformity and a heritability of 0.11 for lack of operculum, in descendants from three industrial batches of breeders at commercial size. And Astorga *et al*. (2004) estimated a high heritability for the presence/absence of any type of deformity (0.77 when using an animal model and 0.80 when using a Bayesian model). Both studies were conducted in unsorted fish. Nevertheless, in this experiment, a genetic origin of these deformities is possible as their prevalence was very low, as noted earlier. Deformity is a binary trait, so, when its prevalence is very low, it is difficult to accurately estimate its genetic additive value (Falconer and Mackay, 1996). In fact, Kause *et al*. (2007), in farmed salmonids, estimated close-to-zero values of heritability for skeletal deformities when their prevalence was low and elevated values when their prevalence was high. In this regard, Evans and Neff (2009) did not found a significant relationship between the spinal deformity prevalence and the additive genetic effect in two different populations of chinook salmon (*Oncorhynchus tshawytscha*) larvae, whose average prevalence were close to zero for these deformities in both populations (0.69% and 0.05%).

Considering all this, an efficient strategy to increase the prevalence of deformities and to reveal the genetic origin of the deformities, if that exists, would be to conduct an intentional mating using deformed fish to evaluate their effective predisposition to produce

deformed progenies (Bardon *et al*., 2009). This statement led us to Experiment 3, in which directed matings formed by two deformed sires (lack of operculum, lordosis and vertebral fusion) and by one non-deformed dam were conducted. A mass spawning control was considered. A significant relationship between the prevalence of a deformity and the mating of breeders suffering from the same deformity was obtained after evaluating the 11503 individuals.

However, a statistically significant prevalence of lack of operculum was observed in both NxO and NxL matings, suggesting that it is not related to family structure. Additionally, the family (or replica) effect in the NxO mating was significant, thus, just one family was responsible for the high prevalence of operculum deformity in this mating. These results are similar to those obtained in Experiment 2 and to that obtained by Lee-Montero *et al*. (2014), as offspring evaluation was conducted at a small size (129 days post-hatching). On the other hand, no significant prevalence of lordosis or vertebral fusion was observed in the other matings. It is also remarkable the fact that the number of normal individuals was significantly higher in the control mating, which was formed by non-deformed breeders. Moreover, no tank effect was observed, as expected from the fact that the rearing conditions were similar in all the matings. The replica effect could not be analyzed in matings NxL and NxVF because of their high larvae mortality, presumably due to a lower viability in matings in which one of the breeders shows these deformities. All these results support the idea that the column deformities show an important genetic determination.

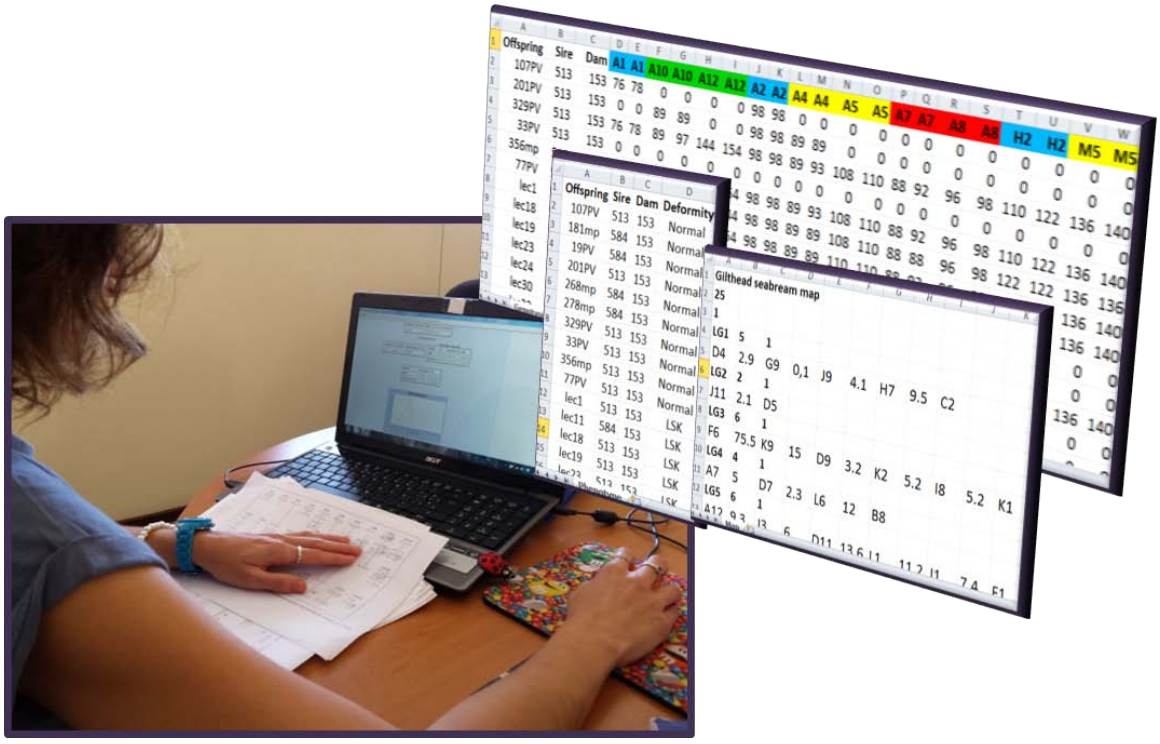
When comparing the deformities prevalence among the experiments (Table 4), it can be observed that LSK deformity presents the lowest prevalence in all the offspring (0-0.2%), revealing that it is an extremely rare deformity. That could be the reason by which it has been rarely included in deformities studies (Afonso *et al*., 2000; Ebrahimnezhad *et al*., 2009). This extremely low prevalence could be also because, being a severe deformity, it is easier to be

diagnosed at early ages, so that hatcheries can detect and eliminate them before the fish sale to on-growing companies, entailing economic losses. In addition, as it has been observed in this study, mortality of these individuals is high even when they are separated from non-deformed fish, so, it can be deduced that these individuals do not reach their final on-growing stage. In Experiment 1, by using non-deformed breeders and culling deformed individuals, the prevalence of deformities at the final point was 12.55%, more concretely; column deformities prevalences were higher than operculum deformities prevalence. Similarly, Oliva (2008) observed, in an industrial batch of 430 g gilthead seabream, a 9.80% of the fish with vertebral deformity and a 0.8% of head and operculum deformities. Contrastly, in Experiment 2, despite no sorting process was carried out, the column deformity prevalence was lower than in Experiment 1. It could due to differences among batches, which has already been found by several authors (Witten *et al*., 2009; reviewed by Boglione *et al*., 2013). This highlights that the sorting process is not an efficient method to cull deformed fish from the production. With respect to Experiment 3, the deformities prevalence of descendants from deformed breeders was significantly higher than in control mating descendants and than in Experiment 2 at the initial size. In fact, in Experiment 3, the presence of fish showing column deformity was easily detected even at an early age, possibly due to a higher intensity or severity of that deformity (reviewed by Bardon *et al*., 2009). Differently, in Experiments 1 and 2, deformed individuals were not detected until they reached a higher size.

Conclusion

The results of this study confirm that an important part of the phenotypic variation depends on non-genetic or environmental factors and suggest that producers should control these environmental factors in order to minimize the prevalence of deformities. They also show that the prevalence of the studied deformities (lack of operculum, lordosis, vertebral fusion and LSK complex) significantly correlates with the family structure in the three studied populations. So, to improve significantly the fish morphological quality, it is essential to

know the breeders genetic predisposition to produce deformed descendants, as well as to management optimally breeders during their spawning process.



4. Quantitative Trait Loci for Skeletal Deformities in Gilthead Seabream (*Sparus aurata*, L.); Lack of Operculum, Lordosis, Vertebral Fusion and LSK complex

Negrín-Báez *et al*. (2014c). Genetics. In preparation.

4.1. Abstract

Morphological abnormalities, especially skeletal deformities, are some of the most important problems affecting aquaculture industry. In this work, a QTL analysis for five skeletal deformities in gilthead seabream (*Sparus aurata* L.) is reported. A total of three studies were conducted. All the offspring analyzed and their parents were genotyped by using a set of multiplex PCRs (ReM*sa*1-13), which includes 106 microsatellite markers, and a linear regression methodology by the *GridQTL* software was used to perform the QTL analysis. In Study 1, 78 offspring individuals from five families and six breeders (five full-sib families, three maternal and two paternal half-sib families) were analyzed. They had showed a significant association with prevalence of LSK complex (lordosis-scoliosis-kyphosis) deformity in a previous segregation analysis from an industrial mass-spawning. A total of 14 QTLs were identified for this deformity. Four of them (QTLSK2, 5, 10 and 12), located in LG5, 8, 17 and 20, respectively, were considered the most solid ones. They were significant at genome level and showed an extremely large effect (>35%), and the genotype of their closely located markers showed a strongly association with their phenotype. Five of these molecular markers (*BId-39*-T, P96, *Gt57*, *DId-03-T* and *Bt-14-F*) were considered as potential markers linked to this deformity. In Study 2, 142 individuals of six half-sib families from a unique male, which had showed a high significant association with prevalence of lack of operculum in a previous segregation analysis, were selected for QTL mapping. Two of the four QTLs detected for this deformity were significant QTLs (QTLOP1 and QTLOP2) and they were located in LG9 and LG10, respectively. Both QTLs showed a large effect (about 25%), and a significant association between lack of operculum deformity and male allelic segregation was observed in the QTLOP1. Their confidence intervals were very large, so a finer mapping would be required to more exactly establish the QTL positions. In Study 3, a full-sib family

with 152 descendants from a mating of deformed breeders (lordosis) was analyzed. Seven QTLs were identified for different deformities. Three of them were significant: one for vertebral fusion (QTLFV3) in LG21, one for lordosis (QTLORD1) in LG9 and one for jaw deformity (QTLJW1) in LG13. Close markers analyzed for QTLFV3 (*Hd-46-T*) and QTLJW1 (*CId-26-H* and *CId-03-F*) showed a significant association between a breeder allele and the phenotype. These QTLs were detected in only one family, so they should be confirmed in other gilthead seabream families.

This work reports, for the first time in gilthead seabream, the identification of QTLs related with skeletal deformities, which supposes a critical step in Marker Assisted Selection implementation in this species.

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4.2. Introduction

In aquaculture industry, the presence of morphological abnormalities in fish entails important economic losses. In gilthead seabream (*Sparus aurata* L.) industry, in which products are mainly commercialized as whole fish, deformed fish affect negatively to the turnover of hatcheries and on-growing companies. Because of this, the incidence of anomalies in juveniles from hatchery companies, which ranges currently from 15 to 50%, has to be reduced to 5% prior to batch commercialization (Afonso and Roo, 2007; Prestinicola *et al.*, 2013). This reduction requires individual manual sorting and visual assessment, which introduces an additional cost of about 10% per sold health fingerling (Divanach *et al.*, 1996). However, many abnormalities are detected later and they persist in fish at harvest size. In this way, on-growing companies have to remove deformed fish prior to its commercialization or to sell them at below their production costs, as customers rarely accept fish showing malformations (reviewed by Bardon *et al.*, 2009).

The deformities altering the fish appearance are considered the most important, since they affect directly to their production traits (Afonso and Roo, 2007). Among them, skeletal deformities, such as neurocranium or head, vertebral column and appendicular skeleton, are the most relevant deformities. Head deformities include those affecting jaw and operculum complex (lack of operculum). Jaw abnormalities consist of torsion of the upper or the lower jaw or extension of this in different magnitud, that have been associated with lethal effects (Afonso and Roo, 2007). The lack of operculum is the most common external abnormality in gilthead seabream, affecting up to 80% of the reared population (Galeotti *et al.*, 2000). Although this abnormality does not affect directly to the growth traits, it has been related with lower fish resistance to environment stress and higher fish predisposition to bacterial infections of gills. The lack of operculum damages the final image of the product because of

the exposure of the gills and, in consequence, decreases its commercial value (Beraldo and Canavese, 2011). Lordosis, scoliosis, kyphosis and vertebral fusion are the most frequent vertebral column anomalies (Afonso *et al.*, 2000; reviewed by Boglione *et al.*, 2013), which not only affect the fish appearance, but they lead to physiological alterations that result in a decrease of their commercial traits value: a lower growth rate, a higher mortality during handling and an increased difficulty of filleting. Furthermore, the effect of these anomalies in animal welfare must be also considered (Gjerde *et al.*, 2005; Karahan *et al.*, 2013).

A high number of studies has determined that physiological, environmental, xenobiotic and nutritional factors are linked to fish deformities appearance (Bardon *et al.*, 2009), but an increasing number of studies also consider a genetic origin. In gilthead seabream, a significant association was found between a family and the prevalence of LSK complex (lordosis-scoliosis-kyphosis) by Afonso *et al.* (2000). Other significant associations were also described by Negrín-Báez *et al.* (2014b) between families or breeders and the prevalence of lordosis, vertebral fusion, lack of operculum and LSK complex under different breeding conditions, phenotype of breeders and offspring handling. In the context of PROGENSA® (a Spanish Breeding Program at national scale), heritabilities from 0.06 to 0.11 for lack of operculum and from 0.16 to 0.41 for any type of vertebral deformity were estimated in fish reared under different production systems in four Spanish regions (Lee-Montero *et al.*, 2014). All this suggests that the prevalence of skeletal deformities in this species is determined, not only by environmental factors but also by genetic factors.

The development of genetic tools has allowed researchers to analyze and identify genomic regions that are associated with a specific trait of interest (QTL, Quantitative Trait Loci) and variations of this region that are responsible for phenotypic variations (Doerge, 2002). Having an accurate linkage map is crucial for the searching for QTLs, where the density of genetic markers is an important factor to detect them (Rodriguez-Ramilo *et al.*,

2014). In gilthead seabream, a linkage map based on 204 microsatellite markers and 26 linkage groups (LG) is available (Franch *et al.*, 2006). Microsatellites are probably the most useful molecular markers for medium-high density maps. Multiplex PCR is a highly effective tool to reduce cost per reaction and minimize genotyping errors by reducing steps and introducing automation during the sample analysis process (Navarro *et al.*, 2008). This is especially important in methodologies where a large number of markers has to be genotyped, such as searching QTLs. In gilthead seabream, a set of 13 multiplex PCR assays formed by 106 specific microsatellite markers that cover all LG in the gilthead seabream genetic map have been developed by Negrín-Báez *et al.* (2014a).

Marker-assisted selection (MAS) may be implemented through identification of QTLs. The advantages of MAS are notable as compared with the traditional selective breeding. MAS selection would be especially profitable for traits that are difficult to measure on offspring, that exhibit low heritability and/or that are expressed later in development (Yue, 2014), such as skeletal deformities. The detection and classification of this type of anomalies is very difficult because of the lack of a standardized classification system for reared fish, mainly, at small size. Moreover, estimated heritability for presence/absence of any kind of vertebral deformities and lack of operculum in gilthead seabream showed medium-low values, and was close to zero in small size (Lee-Montero *et al.*, 2014).

Currently, MAS has not played a major role in most of genetic improvement programs in aquaculture industry (Yue, 2014). However, a wide number of studies using microsatellite markers have revealed the existence of QTLs for the most interesting traits in different species. Among these, growth and morphometric parameters (total length, standard length, eyes diameter and eyes cross) were related to QTLs presence in Asian seabass (*Lates calcarifer*), European seabass (*Dicentrarchus labrax*), rainbow trout (*Oncorhynchus mykiss*), turbot (*Scophthalmus maximus*), Atlantic salmon (*Salmo salar*), Artic charr (*Salvelinus*

alpinus) and common carp (*Cyprinus carpio* L.). QTLs related with resistance to microorganism infection and survival have been reported to Furunculosis in turbot and to Infectious Pancreatic Necrosis in Atlantic salmon and rainbow trout. In addition, other traits with an increasing interest, like stress response and salinity tolerance, have shown association to QTLs in European sea bass and salmonids (reviewed by Yue, 2014). In gilthead seabream, several QTLs related with commercial traits have been also reported. Growth, sex determination and several morphometric traits are influenced in this species by 4, 6 and 3 QTLs, respectively, (Loukovitis *et al.*, 2011, 2012 and 2013). Two QTLs were found in GL13 and GL21 for resistance to Pasteurellosis (Massault *et al.*, 2010), and Boulton *et al.* (2011) detected a significant QTL for morphometric traits and two suggestive QTLs for stress response to confinement.

The main objective of the present study was to analyze and scan in 12 families of gilthead seabream for QTLs affecting skeletal deformities; LSK complex, lack of operculum, jaw deformity, vertebral fusion and lordosis.

4.3. Material and Methods

4.3.1. Fish, Trait Measurement and Parental Assignment

Study 1

Mating structure, rearing conditions, phenotypic assessment and parental assignment were previously described in detail in Negrín-Báez *et al.* (2014b). Offspring from an industrial broodstock were reared until 509 days post-hatching (419.7 ± 3.1 g) (mean ± standard error). At 111 days fish were sorted by deformity. Deformed fish were culled with the exception of LSK (lordosis/scoliosis/kyphosis complex) fish that were selected and reared separately. Pedigree was determined by genetic characterization using the microsatellite multiplex PCR SMsa1 (*SuperMultiplex Sparus aurata*) (Lee- Montero *et al*., 2013). A total of 900 fish were phenotypically and genetically analyzed. Out of 89 full-sibling families represented, only six of them, originated from two sires (♂1 $_L$ and ♂2_L) and five dams (♀1 $_L$, ♀2_L, ♀3_L, ♀5_L and ♀6_L), included the total of LSK individuals (Negrín-Báez *et al.*, 2014b). Five of these six families (FAM1 to FAM5) were selected for QTL mapping. Total individuals per family and their phenotype are showed in Table 8.

Study 2

Mating structure, rearing conditions, phenotypic assessment and parental assignment were previously described in detail in Negrín-Báez *et al.* (2014b). Offspring from an industrial broodstock and belonging to the PROGENSA® breeding program (Afonso *et al.*, 2012) were reared until 689 days post-hatching (524.4 ± 12.6 g). A total of 810 fish were phenotypically and genetically analyzed. The pedigree was determined as described in the Study 1 and 66 full-sibling families were formed. Only the operculum deformity prevalence showed any significant statistically association with any breeders or families: one sire, one dam and two families. About 50 % of individuals with lack of operculum were descendant of

this sire (♂1 ♀) (Negrín-Báez *et al.*, 2014b). Therefore, all descendants of this sire (six half-sib families, FAM6 to FAM11) were selected for QTL mapping. Total individuals per family analyzed and their phenotype are showed in Table 8.

Study 3

Fish were obtained from a mating established with two deformed breeders (lordosis). These breeders were from F1 established with deformed sire and normal dams described extensively by Negrín-Báez *et al.* (2014b). Fish were cultured in the industrial-like facilities of the Marine Science and Technology Park of the University of Las Palmas de Gran Canaria (PCTM-ULPGC, Gran Canaria, Spain) in intensive rearing conditions described by Roo *et al.* (2009). At 120 days old (3.74±1.24 g), fish were slaughtered and an initial visual phenotypic analysis was carried out to determine the presence of any type of deformity. Three types of deformities were observed: vertebral fusion, lordosis and jaw deformity. A sample of 152 offspring (FAM12) were selected for QTL mapping and deformities detected in the initial analysis were re-analyzed with soft X-ray monitored (Mod. Senographer-DHR, General electric´s USA). The real proportion of abnormal fish and all types of deformities detected are shown in Table 8.

4.3.2. Genotyping

Offspring and their parents from three analyses were genotyped by using 13 multiplex PCRs (ReM*sa*1 to ReM*sa*13) described in Negrín-Báez *et al.* (2014a). These multiplex PCRs include a total of 106 microsatellite markers which are all located on the genetic map of this species (Franch *et al.*, 2006; Senger *et al.*, 2006).

4.3.3. QTL mapping

A linear regression (LR) methodology (Knott *et al.*, 1996) was used for the detection of QTLs in all analyses. It was performed by using the *GridQTL* software (http://www.gridqtl.org.uk) (Seaton *et al.*, 2006). Due to family structure, a half-sib (HS) and

full-sib (FS) regression analysis was carried out in the Studies 1 and 2, while only full-sib regression analysis was possible in the Study 3. In both analyses, chromosome-wide and genome-wide significance thresholds were estimated by implementing a bootstrapping method at p = 0.05 and p = 0.01 with a permutation test set to 10,000 iterations (Churchill and Doerge *et al.*, 1994). According to chromosome-wide level, QTL was considered suggestive when significance was between 5% and 1% and significant when it was below 1%. According to genome-wide level, QTL was significant when it was between 5% and 1% and highly significant when it was below 1% (Rodriguez- Ramilo *et al.*, 2011; Vallejo *et al.*, 2014). No fixed factor or covariate was included in the model. Confidence intervals were calculated by bootstrapping the samples 10,000 times (Visscher *et al.*, 1996).

The effect of the QTL in terms of the percentage of variance explained (PVE) was estimated, according to Knott *et al.* (1996), by using the difference between the full and reduced models as follows:

$$PVE_{HS}\ (\%) = 4 \times ((RMS\ red - RMS\ full) / RMS\ red) \times 100$$

$$PVE_{FS}\ (\%) = 2 \times ((RMS\ red - RMS\ full) / RMS\ red) \times 100$$

Where HS is half-sib analysis, FS is full-sib analysis, RMS red is the residual mean square from the reduced model in which all the effects, excluding QTL effect, are fitted and the RMS full is the residual mean square from the model in which all the effects, including QTL effect, are fitted.

The magnitude of the QTL effect according to PVE was established following the classification of Massault *et al*. (2011), as small effect when PVE was less than 5% and large effect when was higher than 10% and were included in the classification: medium effect when PVE was between 5 and 10 % and extremely large when was higher than 35%.

Table 8. Family structure, number of offspring analyzed in each study and number of fish showing each deformity

				Deformities					
Sires	***Dams***	***Families***	***N***	***LSK***	***Operculum***	***Vertebral fusion***	***Lordosis***	***Jaw***	***Normal***
Study 1									
♂1_L	♀1_L	**FAM1**	19	13	-	-	-	-	6
	♀2_L	**FAM2**	25	20	-	-	-	-	5
	♀1_L	**FAM3**	6	2	-	-	-	-	4
♂2_L	♀2_L	**FAM4**	13	8	-	-	-	-	5
	♀3_L	**FAM5**	15	9	-	-	-	-	6
	Total		78	52	-	-	-	-	26
Study 2									
	♀1_O	**FAM6**	13	-	6	-	-	-	7
	♀2_O	**FAM7**	5	-	1	-	-	-	4
♂1_O	♀3_O	**FAM8**	79	-	10	-	-	-	69
	♀4_O	**FAM9**	10	-	1	-	-	-	9
	♀5_O	**FAM10**	6	-	1	-	-	-	5
	♀6_O	**FAM11**	29	-	4	-	-	-	24
	Total		142	-	23	-	-	-	118
Study 3									
♂*1*	♀*1*	**FAM12**	152	-	-	20	42	13	77

N: number of analyzed descendants in each family.

4.3.4. Genotypic Association Analysis

To determine the association between phenotype (normal *vs* abnormal) and allele segregated of microsatellite markers that were close to each significant QTL, contingency tables and Pearson chi-square tests were carried out. To that, alleles were codified to discriminate between those coming from the sire and those coming from the dam (s1, s2, d1, d2). The allelic segregation was tested from the sires and dams separately for each marker. The Pearson chi-square test model was the following:

$$X^2 = \sum_{i=1}^{I} \sum_{j=1}^{J} \frac{(o_{ij} - e_{ij})^2}{e_{ij}}$$

Where *o* was the observed frequency, *e* the expected frequency, *i* the allele value and *j* the category of deformity (normal *vs* abnormal). Significant association was considered when $P \leq 0.05$.

The QTL effect in terms of association between phenotype (normal *vs* abnormal) and genotypes of microsatellite markers that were close to each significant QTL was also determined. A one-way analysis of variance (ANOVA) was performed on the phenotypic values of each family's offspring by using individual genotypes (s1d1, s1d2, s2d1, s2d2). Each ANOVA provided a corrected R^2 value that measured the reduction of the overall phenotypic variance of traits due to the model fitting, thus, providing the proportion of the trait variance predictable for the given marker genotypes.

Both analyses were performed by using the statistical software package SPSS (PASW Statistics v18).

4.4. Results

4.4.1. Genotyping

Out of 106 microsatellite marker genotyped, there were several no informative markers in each family because either the breeders were homozygous or null alleles were observed. The number of informative microsatellite markers per family, map length covered, the average distance between microsatellite markers and the average number of microsatellite markers per linkage group are shown in Table 9.

Table 9. QTL mapping information. Number of microsatellite markers used per family (NMk), map length covered in cM, the average distance between microsatellite markers (MD) and the average number of microsatellite markers per linkage group (MMk) in two types of QTL analysis

Study*	Families	NMk	cM	MD	MMk
Study 1	FAM1	95	936.7	15	3.8
	FAM2	96	886.2	13.8	4
	FAM3	94	932.5	15	3.8
	FAM4	94	952.4	14.9	3.9
	FAM5	94	936.9	15.1	3.9
Study 2	FAM♂1_O	101	916.9	13.8	4
Study 3	FAM12	93	748.5	14.6	3.9

*Data shown in Study 1 and 3 were obtained from full-sib analysis, while data shown in Study 2 were obtained from half-sib analysis

4.4.2. Study 1

QTL Mapping

A full-sib regression analysis was carried out for FAM1-5 and half-sib regression analysis was carried out for families from♂1_L and ♂2_L (paternal half-sib) and families from ♀1_L, ♀2_L and ♀3_L (maternal half-sib). Both analysis, moreover, were carried out at chromosome and genome-wide level. In this study, the QTLs that were significant in, at least, two of the analyses related with family structure were considered solid. Fourteen QTLs for LSK complex deformity were detected and their positions, confidence intervals and

significances from all the analyses are shown in Table 10. Four of them (named QTLSK2, QTLSK 5, QTLSK10 and QTLSK 12) were considered solid, and showed significance also at genome-wide level in, at least, one analysis. The percentages of variance explained by solid QTLs (PVE) are shown in Table 13.

Genotypic Association Analysis

All microsatellite markers from LG where a solid QTL for LSK complex deformity was detected were analyzed, although only data from those that were closer to each QTL are shown. Microsatellite markers, their allelic significant association for breeders and the percentage of variance explained by genotype (R^2) are shown in Table 13.

Markers *BId-39-T* (28.9 cM) and *P96* (40.1 cM) were the closest marker to QTLSK2 and a significant association between the allelic segregation of a dam and LSK deformed offspring was found. All the individuals with LSK complex showed the allele 74 for *BId-39-T* and the allele 78 for *P96*, while none of the normal individuals showed these alleles. In LG8 the closet marker for QTLSK5 was DId-22-F, which showed a significant association between the allelic segregation of a dam and LSK deformed individual (P=0.05). Only a microsatellite marker was close to QTLSK10, *Gt57* (0 cM). Significant Pearson chi-square test revealed a relationship between the allelic segregation of a sire and a dam and their LSK offspring ($P<5\cdot10^{-4}$ and $P\leq4\cdot10^{-3}$, respectively). For this marker, LSK individuals inherited the allele 150, while normal individual did no show this allele.

The closest microsatellite markers for QTLSK12 position were *DId-03-T* and *Bt-14-F* (1.3 cM). For *DId-03-T*, the use of contingence tables and a significant Pearson X^2 test showed a high association of breeder's allelic segregation for this marker with LSK offspring, both sires ($P\leq4\cdot10^{-5}$) and dams ($P\leq0.01$). All the individuals with this deformity inherited the allele 126 from sire or from dam, while none of the normal fish inherited this allele. Similar results were observed for *Bt-14-F*, with a high significant association between LSK and allelic

segregation of sires ($P \leq 0.01$) or dams ($P < 5.10^{-3}$). In this case, all the LSK offspring showed allele 110, which did not appear in normal individuals.

4.4.3. Study 2

QTL Mapping

Results revealed two significant QTLs in LG9 (QTLOP1) and LG10 (QTLOP2) and two suggestive QTLs in LG12 (QTLOP3) and LG14 (QTLOP4), with respect to the lack of operculum deformity (Table 11). Half-sib regression for descendants from the sire ♂1 ○ was the most robust analysis because of the structure familiar design, so the significant QTLs from this analysis were considered solid. The percentage of variance explained (PVE) by these solid QTLs are shown in Table 13.

Genotypic Association Analysis

Closely located microsatellite markers, their allelic significant association for breeders and percentage of variance explained by genotype (R^2) for each solid QTL are shown in Table 13. The microsatellite markers closely located to QTLOP1 position were *CId-89-H* (26.7 cM) and *Gd-78-F* (40.5 cM), however, *CId-89-H* was an uninformative marker because breeders were homozygous for this *locus*. With respect to Gd-78-F, Pearson chi-square test showed a significant association (P value= 0.02) between dam allelic segregation and presence of the deformity in offspring. No significant association with closely located markers was found for QTLOP2.

Table 10. Description of QTL detected in Study 1 for LSK complex in gilthead seabream: type of analysis (FS, full-sib; pHS, paternal half-sib; and mHS, maternal half-sib), position in cM, 95% confidence interval in cM (CI) and statistical value for QTL (F)

			Analysis		
QTL	**LG**		**FS**	**pHS**	**mHS**
QTLSK1	1	**Position**			0
		CI			0-16
		F			3.6*
QTLSK2	5	**Position**	39		17
		CI	11-45		0-47
		F	25.1**$^{+}$		6**
QTLSK3	6	**Position**			25
		CI			3-45
		F			3.6*
QTLSK4	7	**Position**			13
		CI			0-56
		F			6.1**
QTLSK5	8	**Position**	29	34	26
		CI	10-36	17-39	3-39
		F	9.1*	8.6**$^{+}$	4.9**
QTLSK6	10	**Position**			46
		F			3.8*
		CI			0-46
QTLSK7	11	**Position**			0
		CI			0-51
		F			3.5*
QTLSK8	12	**Position**	48		
		CI	0-48		
		F	20.4**		
QTLSK9	15	**Position**		9	
		CI		0-9	
		F		4*	
QTLSK10	17	**Position**	0	0	7
		CI	0-20	0-17	0-129
		F	147.4**$^{++}$	25.6**$^{++}$	4.3*
QTLSK11	18	**Position**		8	
		CI		0-28	
		F		4.8*	
QTLSK12	20	**Position**	2	0	2
		F	633.6**$^{++}$	51**$^{++}$	30.3**$^{++}$
		CI	1-4	0-10	1-4
QTLSK13	23	**Position**			0
		CI			0-2
		F			6.4**
QTLSK14	25	**Position**		4	
		CI		1-37	
		F		5.4*	

* P≤0.05 at chromosome level (suggestive QTL). **P≤0.01 at chromosome level (significant QTL). +P≤0.05 at genome level (significant QTL). ++P≤0.01 at genome level (highly significant QTL).

Table 11. Description of QTLs detected in Study 2 for lack of operculum deformity in gilthead seabream: type of analysis (FS, full-sib and paternal half-sib), position in cM, 95% confidence interval in cM (CI)and statistic for QTL (F)

			Analysis	
QTL	**LG**		SP	pHS
QTLOP1	9	**Position**	29	17
		CI	21-49	2-84
		F	33.1**	10.9**
QTLOP2	10	**Position**		29
		CI		13-46
		F		10.5**
QTLOP3	12	**Position**		42
		CI		7-44
		F		8.1*
QTLOP4	14	**Position**		84
		CI		3-96
		F		6.1*

* P≤0.05 at chromosome level (suggestive QTL). **P≤0.01 at chromosome level (significant QTL).

4.4.4. Study 3

QTL Mapping

The full-sib regression analysis carried out revealed 7 QTLs (Table 12). Among them, there were a significant QTL for each deformity studied in this analysis. One significant QTL for vertebral fusion was detected in LG21 (QTLFV3), and two suggestive QTLs in LG4 and LG13. With respect to lordosis, one significant QTL was detected in LG9 at chromosome and genome-wide level (QTLORD1) and another suggestive QTL in LG22. Additionally, a significant QTL for jaw deformity was detected (QTLFW1) in LG13 and another suggestive in LG16. The percentage of variance explained (PVE) by all significant QTLs are shown in Table 13.

Genotypic Association Analysis

Close microsatellite markers, their allelic significant association for breeders and percentage of variance phenotypic explained by genotype (R^2) for each significant QTL are shown in Table 13. Two closed mircrosatellite markers were analyzed for QTLFV3, *Hd-46-T*

and *DId-12-F* (0 cM), however, *DId-12-F* was an uninformative marker, because breeders were homozygous for this *locus*. A significant association between sire allelic segregation and vertebral fusion offspring was observed (P≤0.03).

For QTLORD1, the closely located microsatellite markers *Gd-78-F* (40.5 cM) and *CId-89-H* (26.7 cM) were uninformative (same above). No significant association was found between lordotic offspring and breeder's allelic segregation for any microsatellite marker located in LG9.

Table 12.Description of QTL detected in Study 3 for vertebral fusion, lordosis and jaw deformity in gilthead seabream: type of analysis (FS, full-sib), position in cM, 95% confidence interval in cM (CI) and statistic for QTL (F)

			Analysis
QTL	**LG**		**SP**
QTLFV1	4	**Position**	8
		CI	8-16
		F	11.8*
QTLFV2	13	**Position**	12
		CI	7-12
		F	6.3*
QTLFV3	21	**Position**	0
		CI	4-4
		F	4.3**
QTLOR1	9	**Position**	27
		CI	26-27
		F	63.6**$^{+}$
QTLOR2	22	**Position**	0
		CI	-
		F	4.3*
QTLJW1	13	**Position**	11
		CI	11-12
		F	19.1**
QTLJW2	16	**Position**	4
		CI	1-4
		F	16.6*

* P≤0.05 at chromosome level (suggestive QTL). **P≤0.01 at chromosome level (significant QTL). +P≤0.05 at genome level (significant QTL).

Table 13. Percentage of variance explained by the QTL expressed in PVE and R^2 and allelic association analysis for microsatellite markers close to each solid QTL detected in the three studies

Study	QTL	LG	PVE		Marker	IC	X^2(♂)	X^2(♀)	R^2
			HC	MH					
Study 1	QTLSK2	5	7.2	43.6	*BId-39-T*	L1	(-)	♀**3**$_L$ (P< $2\cdot10^{-3}$)	42.4±23.5
					P96	J1	(-)	♀**3**$_L$ (P< $6\cdot10^{-3}$)	30.9±24.7
	QTLSK5	8	2.5	60	*DId-22-F*	M4		♀***1***$_L$ (P=0.05)	30.4±40.4
	QTLSK10	17	36.8	100.8	Gt57	B3	♂***2***$_L$ (P<$5\cdot10^{-4}$)	♀***2***$_L$(P<$4\cdot10^{-3}$)	85.7±40.9
	QTLSK12	20	98.7	220.5	*DId-03-T*	L9	♂***2***$_L$ (P<$4\cdot10^{-5}$)	♀***1***$_L$(P<0.01) ♀***2***$_L$(P<$5\cdot10^{-8}$)	89.8±17.7
					Bt-14-F	A5	♂***1***$_L$ (P<0.01) ♂***2***$_L$ (P<$8\cdot10^{-6}$)	♀***3***$_L$(P<$5\cdot10^{-3}$)	69.4±41.6
Study 2	QTLOP1	9	7.4	26.5	*Gd-78-F*	L10	♂**1**$_o$ (P<0.03)	(-)	3.6
	QTLOP2	10	-	25.3	*BId-15-F*	B7	(-)	(-)	4.3
Study 3	QTLFV3	21	0.1	-	*Hd-46-T*	A4	♂***1*** (P<0.03)	(-)	3.4
	QTLORD1	9	1.4	-	*DId-14-H*	C1	(-)	(-)	1.6
	QTLJW1	13	0.4	-	*CId-26-H*	F2	(-)	♀***1***(P<$8\cdot10^{-5}$)	11.4
					CId-03-F	G2	(-)	♀***1***(P<0.01)	7.8

X^2*:* Pearson chi-square test: significance when P≤0.05); (-): any significance observed

For QTLJW1, the position estimated was 11cM, but this position was calculated by considering as 0cM the position of marker *Bd-29-H* (17.3 cM), which was the first analized in this LG. So, the real position of QTLJW1 was 28.3 cM, and *CId-26-H* (27.9 cM) and *CId-03-F* (30 cM) were its closest microsatellite markers. A significant association ($P \leq 0.01$) for maternal allelic segregation in both markers was obtained by Pearson chi-square test.

4.5. Discussion

The development of new genetic technologies has significantly increased in the last decades. From the emergence of the molecular-based knowledge as a tool in the aquaculture genetic improvement, this has continued to gain importance and it will become increasingly important as aquaculture further develops (Dunham *et al*., 2014). In the present study, genotyping of all families analyzed was conducted by using 106 microsatellite markers from 13 multiplex PCRs located in the linkage map for this species (Franch *et al*., 2006; Senger *et al*., 2006). The use of multiplex PCR allowed the automation of the genotyping process, which entails a significant reduction of costs and minimization of errors (Negrín-Báez *et al*., 2014a). The average space between these microsatellite markers was 14.6 cM, what is below the recommended maximum distance at QTL searching (20 cM) (Massault *et al*., 2008). This distance between markers is larger than in other studies of searching QTLs for this species (Boulton *et al*., 2011; Loukovitis *et al*., 2011, 2012 and 2013). However, it must be noted that, in the present study, an average of 72.6% (901.4 cM) of total length of the genetic linkage map (1241.9 cM) of gilthead seabream has been covered; while in the other studies, the covered length was significantly lower: 495.4 cM by Loukovitis *et al*. (2012) or 472 cM by Boulton *et al*. (2011).

In Aquaculture, QTL mapping studies that are based on detection in F1 (full-.sib or half-sib) families have been demonstrated to be successful methods developed for most of marine fish species, since they are prolific and it is easy to obtain large families. In this type of method, sample size, heritability of trait, average allelic substitution effect of the involved alleles and association with closely located markers determine its power to detect the QTLs that are responsible for a specific target trait phenotypic variance (Wang *et al*., 2006). In this way, methodology based on linear regression was used in the present study to detect QTLs by

performing *GridQTL* software, which has been evidenced to be robust for use with discrete characters, including binary traits (Gorman *et al*., 2011), as it is the case of deformities. More concretely, in this study, six families containing from 5 to 152 individuals per family were analyzed and medium-low heretitabilities for deformities had been estimated for this species (Lee-Montero *et al*., 2014).

4.5.1. Study 1

LSK complex is a triple column deformity that is easily diagnosed at early ages and that is presented with a low prevalence in gilthead seabream fingerlings. However, it is very severe and produces high mortality, so hatcheries have to detect and discard the fish suffering from this deformity before selling them to on-growing companies. Thus, it has a substantial economic impact (Negrín-Báez *et al*., 2014b). In this study, for the first time for gilthead seabream, QTLs for this deformity have been identified. A total of five full-sib families (three maternal and two paternal half-sib families) were genotyped and 14 LSK-complex related QTLs were found. The finding of a large number of QTLs suggests that this deformity is influenced by a complex interaction between several regions of the genome, which was expected since this deformity is composed of three different deformities. This is also in concordance with the genetic origin of this complex deformity previously proposed by Afonso *et al*. (2000) and Negrín-Báez *et al*. (2014b), who found significant statistical associations between the prevalence of this deformity and certain specific families in gilthead seabream. Four of the QTLs detected in the present study (QTLSK2, 5, 10 and 12) were considered the most solid ones, since they were significant in, at least, two of the analyses conducted and related with family structure (full-sib and half-sib) at chromosome level. It is remarkable that these 4 QTLs also reached a genome-wide level of significance in, at least, one of the analyses; this is a more stringent statistical level (Loukovitis *et al*., 2012). QTLSK2 (in LG5) and QTLSK5 (in LG8) were significant, and QTLSK10 (in LG17) and QTLSK12 (in LG20) were highly significant. Additionally, according to their percentage of phenotypic

variance explained (PVE), these solid QTLs showed an extremely large effect in half-sib analysis, and QTLSK10 and QTLSK12 also in full-sib analysis. Actually, PVE values of these two QTLs in the half-sib analysis were very high and theoretically impossible, since they were over 100% (100.8 and 220.5%, respectively). It is a common result in QTL mapping studies, as PVE tends to be overestimated (Wang *et al*., 2006). It can be explained by the type of analysis (linear regression versus mixed models) (Rowe *et al*., 2006) and by the Beavis effect (Xu, 2003). The Beavis effect explains that the detected QTLs effect is overestimated because QTL identification requires a genetic effect estimation and a statistical analysis in a shorten distribution; and the overestimated QTL effect values can be lower as offspring size is larger (Xu, 2003). In this study, families sizes ranged from 6 to 25 individual per full-sib family and from 15 to 44 per half-sib family, so QTL effect values could be closer to real magnitude when analyzing larger families. Anyway, it is clear that the effect of the four solid detected QTLs is extremely large, especially QTLSK10 and QTLSK12. In fact, they are the only QTLs described in gilthead seabream that have shown an extremely large effect (Massault *et al*., 2010; Boulton *et al*., 2011; Loukovitis *et al*., 2011, 2012, 2013). These results indicate that LSK complex deformity might be controlled mainly by these few large-effect QTLs. In this way, it must be considered that only 78 fish were analyzed, so these QTLs must be solid. This was expected, since the five analyzed families were from a larger experiment in which only six out of 89 families contained the total incidence of LSK individuals, and a significant relationship between breeders and families showing this deformity was obtained (Negrín-Báez *et al*., 2014b).

Moreover, a highly significant association between phenotypic trait and molecular genotype in these solid QTLs for LSK complex was observed. In fact, close microsatellite markers analyzed for these QTLs seem to be linked with LSK complex, so all LSK descendants of, at least, one breeder showed the determined alleles, while normal individuals did not show these alleles, and all LSK descendants of, at least, one full-sib family showed

determined genotypes, while normal individuals did not show them. In fact, the mean values of R^2 for genotypes within full-sib families were also very high (30.4-89.8%). This strong linkage between these solid QTLs and their markers is very important and may have quite repercussions, like the linkage between the marker *Id13* and a QTL for pasteurellosis resistance found by Massault *et al*. (2010), in which all the descendants of a full-sib family that showed a determined allele, died.

With respect to Marker Assisted Selection (MAS), it is a powerful strategy that can help to increase breeding programs gains. Two cases of MAS-including programs has been successfully applied for diseases resistance in aquaculture species like Japanese flounder and Atlantic salmon (Ozaki *et al*., 2012), thus validating this methodology. In this way, in three of this-study solid QTLs (QTLSK2, QTLSK5 and QTLSK10), the 95% confidence intervals were large (between 20 and 47 pb). This could have a negative effect in MAS application since it implicates that marker–QTL linkage phases are not stable over generation or population. This can be solved in future analyses by increasing the family size, by using more microsatellites to reduce the space between markers or by using new markers obtained by next generation sequencing analysis (SNPs) (Loukovitis *et al*., 2012). In this way, Negrín-Báez *et al*. (2014a) described 20 alternative microsatellite markers that were not included in the set of multiplex PCR ReM*sa* but that could be included in any multiplex in a further screening, as their corresponding primers have also been redesigned to be amplified under the same PCR conditions. With respect to QTLSK12, it showed a short 95% confidence interval (4 or 10 pb depending on the analysis), which, taken together with the strong linkage of this QTL and its microsatellite markers, indicates the importance of including this QTL in a MAS selection program. Moreover, in a genetic breeding program of giltheah seabream under industrial conditions, the use of molecular markers to determine the pedigree is necessary (*Navarro et al*., 2008, 2009a, 2009b). In fact, one of the close-to-QTLSK12 markers (*Bt-14-*

F) is included in an international panel for this species (SMsa1 multiplex PCR) (Lee-Montero *et al*., 2013).

In conclusion, the results obtained in this study confirm the genetic origin of this severe skeletal deformity and show four solid QTLs, two of them (QTLSK10 and QTLSK12) being strongly linked with LSK complex, as well as five molecular markers (BId-*39-T*, *P96*, *Gt57*, *DId-03-T* and *Bt-14-F*) as potential candidates to be included in a MAS selection for this species, especially *Bt-14-F* (QTLSK12). Moreover, selecting the appropriate breeders by MAS selection for non-LSK individuals would decrease the prevalence of this deformity in hatcheries and, at the same time, could also decrease the prevalence of another skeletal deformity (lordosis and vertebral fusion), since the results of a previous study of segregation suggested a possible relationship between these deformities (Negrín-Báez *et al*., 2014b), further increasing companies gains.

However, more microsatellite markers for narrow regions of interest and larger families to increase the precision of QTL position detection and its effect should be considered in future studies.

4.5.2. Study 2

The lack of operculum is the most common external abnormality in gilthead seabream and reduces the quality of fish, thus decreasing its market value (Beraldo *et al*., 2003). This deformity is easily detectable at early ages because it is clearly evident and it can be observed from 25 days-old larvae (Boglione *et al*., 2013). There are several studies on fingerlings demonstrating that controlling environmental factors like nutrition (Beraldo *et al*., 2003) or culture conditions (Roo *et al*., 2005; Prestinicola *et al*., 2013) may decrease the prevalence of this deformity but not its elimination. Therefore, hatcheries perform visual assessment and manual sorting to discard abnormal fish prior to selling batches to on-growing companies. Despite this strategy, there are several fish showing this deformity at commercial size (300-600 g). For instance, in gilthead seabream industrial slaughter size batches, it has

been observed a 0.8% and 0.4% of lack of operculum deformed fish, (Oliva, 2008; Negrín-Báez *et al*., 2014b), as this deformity can appear in fish up to 269 days-old (Lee-Montero *et al*., 2014). Other studies suggested that, while the prevalence of lack of operculum in small size fish is mainly influenced by the environment, at larger sizes it has a significant genetic determination (Lee-Montero *et al*., 2014; Negrín-Báez *et al*., 2014b). In consequence, MAS selection could be very helpful to reduce this deformity prevalence in a breeding program, since it is especially useful for traits that are measured later in development (Yue, 2014). In this way, in the present study, for the first time for gilthead seabream, the genetic origin of lack of operculum deformity was confirmed as QTLs for this deformity were identified. Two out the four detected QTLs for this deformity were significant by means of half-sib analysis and were located in LG9 (QTLOP1) and LG10 (QTLOP2). This analysis was considered more robust because the male effect on lack of operculum deformity could be determined by reducing common environmental sources, having been paired with several dams, and because male ♂1O's half-sib family analyzed was selected out after checking a significant association with lack of operculum in descendants from this male at commercial size (Negrín-Báez *et al*., 2014b). In fact, 48.94% of offspring showing lack of operculum in that previous experiment were descendants of this sire. It must be noted that QTLOP1 seems to be a more solid QTL for this deformity, since it was also significant in the full-sib analysis.

These QTLs explain about 25% of phenotypic variation for this deformity, according to their PVE values. This supposes that these QTLs have a large effect, which is a reason to include MAS in breeding programs (Yue, 2014). However, their 95% confidence intervals were very large, so it would be necessary to more exactly establish the QTL positions by using new microsatellite markers with genetic variability for this male in these LGs. In this regard, there are microsatellite markers (7 in LG9 and 3 in LG10) in the genetic linkage map of gilthead seabream (Franch *et al*., 2006; Massault *et al*., 2010) that were not included in the set de multiplex and that could be used to improve the QTL mapping. Nevertheless, the

difficulty to precisely locate QTL may also be attributed to the family low size (Loukovitis *et al*., 2012).

On the other hand, a significant association between operculum deformity and male's allelic segregation was observed only in QTLOP1 for the marker Gd-78-F, thus confirming that this QTL is stronger that others, although its R2 was slightly lower.

In conclusion, the detected QTLs for lack of operculum in this study (QTLOP1 and QTLOP2), evidence the possibility of introducing this trait in a breeding program through MAS selection in this species, mainly QTLOP1, although a finer mapping would be required. MAS selection could mean a reduction of prevalence of the most common external deformity in gilthead seabream and, thus, a decrease of derived economic losses.

4.5.3. Study 3

The present study identifies, for the first time, seven QTLs for three of the most relevant and frequent skeletal deformities from a full-sib family of gilthead seabream.

Lordosis and vertebral fusion are the most important skeletal deformities, as they reduce the fish weight and, subsequently, they strongly decrease the commercial value of slaughtered fish (Afonso *et al*., 2000; Boglione *et al*., 2013). Their prevalence is associated to environmental conditions (reviewed by Boglione *et al*., 2013), but a genetic component has also been proposed (Lee-Montero *et al*., 2014; Negrín-Báez *et al*., 2014b). Even a major QTL for scoliosis has been described in guppy (Poecilia reticulata), which contains the MTNR1B, a candidate gene for human idiopathic scoliosis (Gorman *et al*., 2011). For vertebral fusion, one of the three QTLs detected in this study was significant (QTLFV3) and was located in LG21. Its effect was small, according to its PVE, however, it was located in a short LG, so association between markers and detected QTLs should be more powerful (Massault *et al*., 2010). In this way, its close marker analyzed (Hd-46-T) showed a significant association between male's allelic segregation and presence of the deformity in offspring, additionally, its genotype explained the 3.6% of the phenotypic variance. One of the two QTLs found for

lordosis (QTLORD1) was significant and was located in LG9. It was remarkable that this QTL was significant also at genome level, which is a more stringent statistical level (Loukovitis *et al*., 2012). However, its effect, in terms of PVE, was also small and no significant association was detected between the marker genotypes of its LG and presence of the deformity. On the other hand, as the solid QTL for lack of operculum locates also in this LG, if a finer mapping was carried out in a new population, lordosis could also be measured and analyzed. This QTL, like the one for vertebral fusion, confirmed the genetic origin of these spinal deformities.

Jaw deformity is also a frequent skeletal deformity, although it entails a lower economic impact. Many studies related its prevalence with environmental conditions (Boglione *et al*., 2013), but not with genetic factors. In fact, Lee-Montero *et al*. (2014) estimated a heritability close to zero for head deformities, however, this can be explained because, beside jaw deformities, they included other head anomalies more difficult to evaluate and that could increase the environmental variance. On the contrary, a significant QTL for jaw deformity was located in LG13 (QTLJW1), as well as a suggestive one in LG16. According to PVE, the effect of QTLJW1 was also small, however, a significant association between female's allelic segregation and presence of the deformity was detected in two close markers (CId-26-H and CId-03-F). More concretely, the genotype of both markers explained the 11.4% and 7.8% of phenotypic variance. This suggests that, although this QTL showed a small effect, it could be located near the analyzed markers. In fact, both markers are separated 2.1 pb according to the genetic map (Franch *et al*., 2006). The detection of this QTL evidences, for the first time for gilthead seabream, that jaw deformity is determined genetically, in addition to being influenced by environmental factors.

On the other hand, the three significant QTLs of this study were detected by using a unique full-sib family. Nevertheless, this method has some limitations: the found QTLs only

segregate in two breeders and they could be not representative for other gilthead seabream families (Wang *et al*., 2006; Ruan *et al*., 2010).

In conclusion, the results of the present study show the first QTLs identified for lordosis, vertebral fusion and jaw deformity in gilthead seabream, which could help to reduce these deformities prevalence in future by MAS selecting breeding and to solve one of the most important problems in aquaculture. However, these QTLs should be confirmed in other gilthead seabream families before using them.

5. CONCLUSIONS

1. A set of 13 multiplex PCRs (named from ReM*sa*1 to ReM*sa*13) has been developed. They include 106 microsatellite markers of gilthead seabream genetic map that cover 100% of the linkage groups. This battery is a powerful tool for QTLs searching in this species.
2. Twenty additional microsatellite markers have been redesigned and evaluated. Their amplification occurs under the same PCR conditions of set ReM*sa*, and they can be included in analyses in future.
3. It has been determined that the skeletal deformities LSK complex, lordosis and vertebral fusion have a genetic origin in gilthead seabream, as they showed a significant association with family structure.
4. The high prevalence of lordosis and vertebral fusion deformities in descendants from LSK-complex breeders suggests that a relationship between these three deformities exists in gilthead seabream.
5. Lack of operculum deformity in this species is mainly influenced by environment factors in small size fish, but its genetic determination at commercial size has been confirmed.
6. Both, genetic and phenotypic, controls of gilthead seabream breeders in designed matings affect the quality of spawning in terms of viability and prevalence of deformities.
7. Fourteen QTLs have been related with LSK complex deformity in gilthead seabream. Four of them (those located in linkage groups 5, 8, 17 and 20) were considered the most solid ones, as they showed a very high responsibility in the phenotypic variance for this deformity.
8. Genotype of five microsatellite markers closely located to these four LSK-complex solid QTLs showed a strong association with presence of this deformity in offspring.

So, they have been proposed as potential candidates to be included in a Marker Assisted Selection for this species.

9. Two significant QTLs related to lack of operculum deformity in gilthead seabream were identified. They were located in linkage groups LG9 and LG10 and showed a medium effect on phenotypic variance.

10. A full-sib family of gilthead seabream showed one significant QTL for each one of the following analyzed deformities: lordosis (located in linkage group 9), vertebral fusion (located in linkage group 21) and jaw deformity (located in linkage group 13). However, they should be confirmed in other families.

11. For the first time in gilthead seabream, the genetic origin of jaw deformity has been demonstrated.

12. These results represent a major step towards the location of genes determining the presence of the analyzed skeletal deformities in gilthead seabream, and, subsequently, they offer a useful tool to reduce the prevalence of these deformities when included within a genetic breeding program in gilthead seabream industry.

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ANEXO
RESUMEN EN ESPAÑOL

(EXIGIDO EN EL REGLAMENTO PARA LA ELABORACIÓN, TRIBUNAL, DEFENSA Y EVALUACIÓN DE TESIS DOCTORALES DE LA UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA)

RESUMEN

El objetivo del presente trabajo fue analizar la existencia de QTLs (*Quantitative Trait Loci*) que afecten a las deformidades esqueléticas más relevantes en dorada (*Sparus aurata*), que pudieran ser utilizados como herramienta para minimizar la prevalencia de estas deformidades dentro de un programa de mejora para esta especie. La incidencia de estas anomalías en esta especie supone importantes pérdidas económicas en la industria, tanto en las empresas de cría como en las de engorde, por lo que los peces deformes se eliminan mediante cribas manuales en diferentes puntos de la línea de producción.

En un primer paso, se desarrolló una batería de multiplex PCRs a partir de 138 marcadores microsatélites del mapa genético de la dorada, que se rediseñaron para ser amplificados en las mismas condiciones de PCR. 13 nuevas multiplex PCR (nombradas como ReM*sa*1 a ReM*sa*13) formaron el set con 106 de estos microsatélites, cubriendo el 100% de los grupos de ligamiento. Este set se validó satisfactoriamente, constituyendo una poderosa herramienta para buscar QTLs en esta especie.

Con el fin de encontrar una estructura familiar que facilitara la localización de QTLs, se analizó, en distintas poblaciones de dorada, la prevalencia de cuatro de las deformidades esqueléticas más importantes y graves: ausencia de opérculo, lordosis, fusión de vértebra y complejo LSK (lordosis-escoliosis-cifosis). Para ello se realizaron 3 experimentos a gran escala. En el Experimento 1, se realizó una puesta masal y la descendencia, a los 111 días, se cribó en términos de deformidades. A los 509 días de edad, se analizaron 900 peces: 846 criados en una granja y 54 peces LSK seleccionados en el proceso inicial de criba y criados por separado en un tanque. Todos los individuos que presentaron esta deformidad estaban en seis de las 89 familias representadas, mostrando éstas una asociación significativa con la prevalencia de la deformidad. Por lo tanto, cinco de estas seis familias se seleccionaron para el mapeo de QTL. En el Experimento 2, los peces procedían también de una puesta

masal pero no se realizó ningún tipo de criba. Aunque a talla pequeña la prevalencia de ausencia de opérculo en la descendencia fue independiente de la familia o reproductor, a los 539 días, sí se detectaron asociaciones significativas entre un padre, una madre, y dos de las familias, con la prevalencia de esta deformidad. De hecho, el 48,94% de los individuos que presentaron esta deformidad fueron descendientes de este padre. Por lo que todos los descendientes de este macho (seis familias de medios hermanos) se seleccionaron para el mapeo de QTL. En el Experimento 3, se realizaron cruces dirigidos entre machos deformes que presentaban lordosis, fusión de vértebras o ausencia de opérculo con hembras no deformes, además de una puesta masal como control. A los 129 días un total de 11.503 alevines se analizaron visualmente para las deformidades. Los resultados mostraron una asociación significativa entre la prevalencia de cada deformidad y el cruce en el que el reproductor presentaba la misma deformidad. En los descendientes del cruce del macho lordótico también se observó una significativa prevalencia de falta de opérculo, revelando que esta deformidad parece estar más influenciada a esta edad por factores ambientales, mientras que la lordosis y la deformidad de fusión de vértebras están relacionadas con la estructura familiar.

Por ultimo, se realizó un análisis de QTL para cinco deformidades esqueléticas en 12 familias de la dorada, dividido en tres estudios. Todos los peces se genotiparon utilizando el set de multiplex PCRs ReM*sa*1-13. En el Estudio 1, se analizaron para la deformidad LKS cinco familias seleccionadas en el Experimento 1. Cuatro QTLs significativos, de los 14 encontrados para esta deformidad, situados en los grupos de ligamiento (GL) 5, 8, 17 y 20 presentaron un efecto extremadamente grande y se consideraron los más sólidos. Se observó una fuerte asociación entre el genotipo de los marcadores cercanos a estos QTLs con el fenotipo. En el Estudio 2 se analizó, para la ausencia de opérculo, la familia de medios hermanos paternos seleccionada en el Experimento 2. De los cuatro QTLs detectados para esta deformidad, dos fueron significativos, localizados en el GL 9 y el GL 10, y mostraron un

efecto grande. En uno de ellos se observó una asociación significativa entre la deformidad falta de opérculo y la segregación alélica del macho. En el estudio 3, se analizó para diferentes deformidades esqueléticas, una familia de hermanos carnales procedente de un cruce entre reproductores deformes. Tres de los siete QTLs encontrados fueron significativos, uno para la fusión de vértebras en el GL 21, otro para la lordosis en el GL 9 y otro para la deformidad de mandíbula en el GL 13. No obstante sería recomendable confirmarlos en otras familias de dorada.

Los resultados derivados del presente trabajo, confirman el origen genético de estas deformidades esqueléticas en dorada y su relación con la estructura familiar. Además, se han detectado QTLs para estas deformidades, así como una asociación significativa entre la segregación alélica de los marcadores moleculares cercanos a ellos y el fenotipo. Esto supone un gran paso hacia la localización de los genes que determinan el desarrollo de las deformidades esqueléticas en esta especie. Lo expuesto ofrece a la industria de dorada una herramienta con gran potencial para disminuir la prevalencia de las deformidades dentro de un programa de mejora genética.

1. INTRODUCCIÓN

1.1 Especie de estudio: la dorada

1.1.1 Taxonomía y nomenclatura

La dorada es una especie marina, perteneciente a la familia de los espáridos y al género Sparus (*Sparus aurata*, Linnaeus, 1758). Su clasificación taxonómica es la siguiente:

Reino	*Animal*
Phylum	*Chordata*
Subphylum	*Gnathostomata*
Clase	*Actinopterygii*
Subclase	*Teleostei*
Superorden	*Neognathi*
Orden	*Perciformes*
Familia	*Sparidae*
Género	*Sparus*
Especie	*Sparus aurata*

Sus nombres vernaculares en los distintos idiomas son los siguientes:

Español	*Dorada*
Inglés	*Gilthead seabream*
Francés	*Dorade*
Alemán	*Goldbrasse*
Italiano	*Orata*

1.1.2 Distribución y Hábitat

El área de distribución de esta especie es a través de las costas orientales del océano Atlántico, desde Gran Bretaña hasta Cabo Verde, así como todo el mar Mediterráneo. Debido a sus hábitos eurihalinos y euritérmicos, habita zonas litorales en ambientes marinos, lagunas

costeras y zonas de estuarios, en particular durante las primeras fases del ciclo de vida. Las doradas hacen una migración trófica, desovan en el mar en invierno, posteriormente los juveniles migran a principios de primavera en busca de lugares más protegidos, con abundancia de comida y con temperaturas medias; para luego regresar a mar abierto a finales de otoño para reproducirse (Moretti *et al*., 1999).

1.1.3 Anatomía

La dorada es un teleósteo de cuerpo oval, más bien profundo y comprimido. El perfil de la cabeza aparece regularmente curvado y tiene los ojos pequeños. La boca es baja, levemente oblicua y con labios gruesos. Presenta de cuatro a 6 dientes anteriores de tipo caninos en cada mandíbula, seguidos posteriormente por dientes romos, los cuales se transforman progresivamente a molares y se disponen en 2 a 4 filas (los dientes en las 2 filas externas más fuertes). Presenta un color gris plateado; una gran mancha negra, en el origen de la línea lateral, extendiéndose sobre el margen superior del opérculo. Presenta una banda frontal dorada característica entre los ojos, bordeada por dos áreas oscuras (no bien definidas en los individuos jóvenes); líneas longitudinales oscuras a menudo presentes sobre los costados del cuerpo; una banda oscura sobre la aleta dorsal; así como la horquilla y las puntas de la aleta caudal bordeadas con negro (Figura 1).

1.1.4 Alimentación y Reproducción

La dorada es un pez preferentemente carnívoro, depredadora de especies de fondo (bivalvo y gasterópodo), crustáceos y peces pequeños. En general, se considera una especie de crecimiento rápido en la naturaleza, alcanzando los 300 g en el segundo año y aproximadamente los 600 g en el tercero, pudiendo llegar a tener un tamaño de 70 cm y un peso de 5 Kg (Castelló, 1993).

Es una especie hermafrodita proterándrica, que primero madura como macho y a partir del segundo o tercer año se convierte en hembra. Su estación reproductiva tiene lugar durante un periodo relativamente largo, cuando los días son cortos y las horas de luz decrecientes (octubre a diciembre) en el Mediterráneo Occidental (Arias, 1980). En el Mediterráneo Oriental se produce un poco más tarde, entre noviembre y febrero (Ben-Tuvia, 1979). Los huevos son planctónicos, esféricos y transparentes, de aproximadamente 1 mm de diámetro y con una o varias gotas de aceite en su interior, siendo las cantidades puestas de entre 500.000 y 6.000.000 de huevos/kg de hembra (Cejas *et al*., 1992).

Para el desove en época invernal, la dorada migra hacia aguas costeras, ya que son aguas más protegidas, en busca de alimento abundante y temperaturas más suaves (Migración Tropical). A final de otoño vuelve a migrar a mar abierto para comenzar la época de cría en peces adultos.

1.2. Producción Acuícola de dorada

1.2.1 Producción a nivel mundial y europea

Según datos publicados en APROMAR (2014), existe producción de dorada de acuicultura en 19 países, siendo los principales productores Grecia, con aproximadamente 75.000 toneladas (t) (41,7% de la producción total), Turquía con 41.700 t (23,2%) y España con 16.795 t (9,3%) durante el año 2013. Su cultivo se realiza también en Italia, Egipto, Francia, Chipre, Portugal, Croacia, Malta, Túnez y Marruecos, y hay producciones incipientes en Albania, República Dominicana, Marruecos, Emiratos Árabes Unidos, Bosnia, Omán, Libia y Kuwait (Figura 2).

1.2.2 Producción en España

De la producción de peces en este país, la producción de dorada fue la más elevada en 2013 (16.795 t), seguida por la producción de trucha arcoíris (*Oncorhynchus mykiss*) (16.732 t) y lubina (*Dicentrarchus labrax*) (14.707 t). (APROMAR, 2014).

En 2013, la producción de dorada de acuicultura en España fue liderada por la Comunidad Valenciana con 6.974 t (el 42% del total), seguida por Murcia (3.730 t, el 22%), Canarias (3.016 t, el 18%), Andalucía (1.786 t, el 11%) y Cataluña (1.292 t, 8%) (APROMAR, 2014). En la Figura 3, se muestra la producción de dorada en diferentes comunidades autónomas de España. En la Figura 4 se muestra la producción acuícola total de dorada en Canarias, cuyos datos fueron facilitados por el Ministerio de Agricultura,

Alimentación y Medio Ambiente, Secretaría General de Pesca, Junta Nacional Asesora de Cultivos Marinos (JACUMAR, 2014).

En cuanto a la producción de juveniles en España, el número de unidades asciende a 51,4 millones, lo que supone una reducción del 6,5% sobre el dato de 2012. Esta producción de alevines de dorada en España se concentra en la Comunidad Valenciana, Islas Baleares, Cantabria y Andalucía. Sin embargo, para la producción española de dorada de talla comercial es necesaria la importación de juveniles adicionales a éstos de producción propia. El valor de primera venta a talla comercial de dorada en España en el año 2012 fue de 72.165.279 € (JACUMAR, 2014).

1.3 Mejora genética en dorada

Actualmente, la dorada de crianza supone un 95,3% del total de esta especie consumida en este país. Este marcado desarrollo y expansión de la industria acuícola en torno a la dorada, ha supuesto una alta competencia en el mercado que se ha traducido en un menor precio de venta al consumidor. Por lo tanto, las empresas se han visto obligadas a minimizar costes y aumentar la producción, para así poder mantener los beneficios. En este sentido, la selección genética ha sido reconocida como un importante factor para el desarrollo de una Acuicultura sostenible, eficiente y rentable (Gjedrem, 2005; Rye *et al*., 2010).

Navarro *et al*. (2009a y 2009b) estimaron heredabilidades bajo condiciones industriales, a partir de doradas cultivadas en Canarias tanto en tanques como en jaulas insulares y desarrollaron las herramientas genéticas y protocolos necesarios para implementar

un programa de mejora genética en esta especie sin interferir con el funcionamiento de la industria. Este modelo fue recientemente implementado por el proyecto PROGENSA®, donde se ha desarrollado a escala industrial un programa piloto de mejora en dorada con diferentes empresas de engorde y centros de investigación de cuatro comunidades autónomas, en el que se estimaron parámetros genéticos relacionados con los más importantes caracteres comerciales y establecido la interacción genotipo-ambiente entre comunidades (Afonso *et al*., 2012).

1.4 QTLs

Una forma de mejorar la eficiencia de la selección, dentro de un programa de mejora, es a través de la identificación de QTLs. Un QTL (del anglicismo *Quantitative Trait Loci*) es una región cromosómica (un gen o grupo de genes) que determinan un carácter cuantitativo. La localización de un QTL que afecte a un carácter de marcado interés productivo, permite la identificación del gen o de los genes que afecten a este carácter y con qué intensidad. Esto puede realizarse a través de la Selección Asistida por Marcadores (MAS), aprovechando el desequilibrio de ligamiento entre estos marcadores moleculares y el fenotipo del carácter estudio en la población que se esté analizando (Enciso y Toro, 2007). La detección de QTLs implementada en MAS muestra notables ventajas respecto a un programa de selección tradicional y el uso de esta herramienta se está incrementando cada vez más en la mejora genética animal (Yue, 2014). Para una eficiente detección de QTLs, son necesarios: un mapa

de ligamiento genético con un buen nivel de saturación de marcadores moleculares, datos fenotípicos adecuados y un modelo estadístico genético apropiado (Lynch y Walsh, 1998).

1.5. Marcadores moleculares

En dorada, existe publicado un mapa de ligamiento genético de primera generación basado en 204 marcadores microsatélites y 26 grupos de ligamiento (Franch *et al*., 2006). Los marcadores microsatélites son, probablemente, los marcadores moleculares más útiles para mapas de densidad media-alta, ya que son altamente polimórficos, codominantes, distribuidos a lo largo de los genomas y fácilmente analizables usando la reacción en cadena de polimerasa (PCR) (Bouza *et al*., 2007). Para un QTL determinado, la probabilidad de éxito y resolución del mapeo depende del número de *loci* analizados y la magnitud de su efecto sobre el carácter de interés (Chistiakov *et al*., 2006).

1.6. QTLs en Acuicultura

Actualmente, MAS no ha jugado un papel muy destacado en la mayoría de los programas de mejora genética en la industria de la acuicultura (Yue, 2014). Sin embargo, un gran número de estudios ha revelado la existencia de QTLs para los caracteres más importantes en diferentes especies. Entre éstos, los parámetros morfométricos y el crecimiento (longitud total, longitud estándar, diámetro de los ojos y distancia entre los ojos) han mostrado relación con la presencia de QTLs en lubina asiática o barramundi (*Lates calcarifer*), lubina (*Dicentrarchus labrax*), trucha arcoiris (*Oncorhynchus mykiss*), rodaballo

(*Scophthalmus maximus*), salmón (*Salmo salar*), trucha alpina (*Salvelinus alpinus*) y carpa común (*Cyprinus carpio* L.). También se han descrito QTLs relacionados con resistencia a la infección por microorganismos y supervivencia para Forunculosis en rodaballo, así como necrosis pancreática infecciosa en salmón y trucha arcoiris. Además, otros rasgos con un creciente interés, como respuesta al estrés y tolerancia a salinidad, han mostrado asociación con QTLs en lubina y salmónidos (revisado por Yue, 2014).

En la dorada, también se han descritos varios QTLs relacionados con caracteres de interés comercial en esta especie. Así, Loukovitis *et al*. (2011, 2012 y 2013) encontraron 4, 6 y 3 QTLs para crecimiento, determinación sexual y diversas medidas morfométricas, respectivamente. Para la resistencia a la pasteurelosis también fueron descritos dos QTLs uno en el grupo de ligamiento 13 y otro en el 21 por Massault *et al*. (2010); Finalmente, Boulton *et al*. (2011) detectaron un QTL significativo para medidas morfométricas y dos sugerentes para respuesta al estrés por confinamiento.

1.7. PCR múltiplex

Para la búsqueda y detección de QTLs es necesario un gran número de marcadores microsatélites, con el fin de cubrir el mayor número posible de *loci*, así como un alto número de animales genotipados. Una solución eficaz para reducir el coste por reacción y minimizar los errores de genotipado, son las PCR múltiplex. En este sentido, Navarro *et al*. (2008) demostraron que el costo del genotipado mediante 10 marcadores microsatélites en una PCR múltiplex es una sexta parte del costo del análisis por PCR simple aun si ésta se realizara en

una única carrera electroforética y que el genotipado de mediante PCR múltiplex minimiza errores al reducir pasos y aumentar la automatización durante el proceso de análisis. En dorada, se han desarrollado numerosas PCR múltiplex para determinar las relaciones familiares con un alto nivel de éxito (Launey *et al*., 2003; Brown *et al*., 2005; Navarro *et al*., 2008; Porta *et al*., 2010; Borrell *et al*., 2011). Incluso Lee-Montero *et al*. (2013) propusieron un panel internacional para esta especie compuesto por dos reacciones de PCR múltiplex. Sin embargo, no existen en esta especie PCR multiplex compuestas por un alto número de marcadores microsatélites localizados en el mapa genético adecuados para la búsqueda de QTLs. De hecho, ninguno de los QTLs descritos en dorada y citados anteriormente, han sido detectados usando PCRs múltiplex.

1.8. Las deformidades esqueléticas

1.8.1 Importancia comercial de las deformidades

La presencia de anormalidades morfológicas en los peces cultivados es un problema importante en la acuicultura ya que conlleva importantes pérdidas económicas. Aunque estas pérdidas son difíciles de determinar con precisión, se estima que, en Europa, la cantidad es superior a 50 millones de euros al año, por lo que una reducción del 50% de las deformidades podría ahorrar 25 M€ / año (Hough, 2009). En la industria de la dorada, la cual se comercializa principalmente como pez entero, las deformidades producen altas pérdidas económicas, afectando negativamente tanto a las empresas de cría como a las de engorde. De hecho, los empresarios invierten una cantidad importante de dinero para reducir al máximo la

prevalencia de deformidades, quitando los peces deformes en diferentes puntos del ciclo de producción. De esta manera, un lote de juveniles producidos en una empresa de cría generalmente presenta entre un 15 y un 50% de porcentaje de peces deformes (Prestinicola *et al*., 2013). Por lo tanto, las empresas de cría, deben cribar y desechar peces deformes antes de su comercialización, ya que las empresas de engorde no aceptan más de un 5% de prevalencia (Afonso y Roo, 2007). La criba de peces deformes se realiza mediante un proceso de identificación de las deformidades de manera visual y una clasificación manual e individual, el cual es un método lento y costoso (2 mil peces / hora / persona) (Divanach *et al*., 1996). Esto introduce un coste adicional de aproximadamente el 10% a cada alevín vendido. Sin embargo, muchas anormalidades se detectan más tarde y persisten en los peces en el momento de la cosecha. De esta manera, las empresas de engorde tienen que eliminar los peces deformes antes de su comercialización como pescado entero o venderlos por debajo de su coste de producción, ya que el consumidor rara vez acepta pescado para el consumo que muestre malformaciones (revisado por Bardon *et al*., 2009).

En acuicultura, las deformidades que alteran la apariencia de pescado son consideradas las más importantes, ya que afectan directamente a sus rasgos productivos (Gjerde *et al*., 2005; Kause *et al*., 2005; Afonso y Roo, 2007; Fjelldal *et al*., 2009). Entre ellas, las deformidades esqueléticas, tales como: deformidades cabeza, columna vertebral y esqueleto apendicular, son las deformidades más relevantes. Las deformidades de la cabeza incluyen el complejo opercular (ausencia de opérculo) y las anomalías mandibulares. La ausencia de opérculo es la anormalidad externa más común en dorada, afectando hasta un 80% de las

doradas cultivadas (Galeotti *et al*., 2000). Aunque esta anormalidad no afecta directamente al crecimiento, se ha relacionado con una menor resistencia al estrés ambiental y a una mayor predisposición a las infecciones bacterianas de las branquias. La ausencia de opérculo deteriora la imagen final del producto debido a la exposición de las branquias y, en consecuencia, disminuye el rendimiento comercial de peces (Beraldo y Canavese, 2011). Las anomalías mandibulares, consisten en torsiones de la mandíbula inferior y superior o prolongación de éstas, y en ocasiones han sido asociadas a efectos letales (Afonso y Roo, 2007). Por otro lado, la escoliosis, cifosis, lordosis y fusión vertebral son las anomalías más frecuentes en la columna vertebral (Afonso *et al*., 2000; revisado por Boglione *et al*., 2013). Éstas no sólo afectan al aspecto de los peces disminuyendo su valor comercial y produciendo dificultades en el fileteado, sino que provocan también alteraciones fisiológicas como una menor tasa de crecimiento y una mayor mortalidad durante el manejo que tienen como consecuencia más pérdidas económicas. Sin contar además, el efecto que estas anomalías tienen sobre el bienestar de los animales (Gjerde *et al*., 2005; Karahan *et al*., 2013).

1.8.2. Determinación genética de las deformidades

A pesar la importancia que tienen las deformidades en la industria acuícola y de que suponen una prioridad en las empresas tanto de cría como de engorde, y aunque han sido ampliamente estudiadas, en la actualidad, su etiología y su relación con diferentes etapas del desarrollo no han sido clarificadas completamente. En parte, debido a la falta de un sistema estandarizado de identificación y clasificación para las diferentes anomalías (Boglione *et al*.,

2013), por lo que se hace necesaria la estandarización y homogeneización de éstas (AquaExcel-ATOL, AquaExcel Project, 2013). Por otra parte, la prevalencia de deformidades es diferente entre diferentes empresas de producción, incluso entre los diferentes lotes dentro de la misma empresa. Por lo tanto, es difícil establecer una manera única y eficiente para reducir la incidencia de deformidades en las empresas (Witten *et al*., 2009; revisado por Boglione *et al*., 2013).

Un gran número de estudios han determinado factores fisiológicos, ambientales, xenobióticos y nutricionales vinculados a la aparición de deformidades (revisado por Bardon *et al*., 2009), pero sólo unos pocos de ellos han contribuido a su determinación genética. En dorada, Afonso *et al*. (2000) encontraron una asociación familiar significativa para una deformidad triple de columna en sentido antero-posterior (llamada complejo LSK, lordosis-escoliosis-cifosis) mediante el uso de un esquema de cruzamientos dirigidos. Astorga *et al*. (2004) estimaron la heredabilidad para la presencia/ausencia de cualquier tipo de deformidad en descendientes de reproductores individualmente controlados. Lee-Montero *et al*. (2014), en el contexto de PROGENSA®, estimaron heredabilidades para deformidad en los doradas criadas en diferentes sistemas de producción en cuatro regiones españolas. Los valores variaron de 0,06 a 0,11 para la ausencia de opérculo, y de 0,16 a 0,41 para cualquier tipo de deformidad vertebral. Todos estos estudios sugieren que la variabilidad de las deformidades esqueléticas, además de estar influenciada por el ambiente, también puede explicarse por un origen genético. En este sentido, la estructura del apareamiento parece ser una herramienta adicional e importante a tener en cuenta durante el proceso de control de las deformidades

esquelética tanto a escala industrial como a escala experimental. Además, la selección por MAS sería una estrategia eficaz para optimizar su mejora, ya que es especialmente rentable para los caracteres que son difíciles de medir en la descendencia, que exhiben baja heredabilidad o que aparecen en etapas avanzadas del desarrollo (Yue, 2014), como es el caso de las deformidades esqueléticas. Sin embargo, no existen en dorada ningún QTL descrito para ningún tipo de deformidad.

1.9 Objetivos

En este contexto, el objetivo principal de este trabajo fue: analizar y determinar si existen QTLs que afecten a las deformidades esqueléticas más frecuentes y graves en doradas, que puedan ser útiles para minimizar la prevalencia de estas deformidades en un programa de mejora en esta especie.

Para ello, se determinaron los siguientes objetivos secundarios:

1- Desarrollar un batería de PCRs múltiplex que contuvieran el mayor número de marcadores microsatélites específicos para dorada, con el fin de cubrir la mayor proporción de grupos de ligamiento del mapa genético de esta especie, como una herramienta eficiente para localizar QTLs. Así como evaluar y validar su correcto funcionamiento.

2- Analizar la prevalencia de cuatro deformidades esqueléticas (ausencia de opérculo, lordosis, fusión de vértebra y complejo LSK) y determinar si tiene relación con la estructura familiar, en distintas poblaciones de dorada, con el fin de confirmar el

origen genético de estas deformidades y encontrar una estructura familiar que facilite la búsqueda de QTLs.

3- Genotipar con un set de múltiplex PCRs las familias que tuvieran una asociación significativa con las deformidades analizadas para buscar y localizar posibles QTLs asociados a estas deformidades.

2. MATERIAL Y MÉTODOS

2.1 Diseño de una batería de 13 PCRs múltiplex de marcadores específicos como una herramienta para la detección de QTLs en dorada (*Sparus aurata* L.)

2.1.1 Material biológico

Se analizaron un total de 16 doradas procedentes de tres lotes de reproductores industriales de diferentes comunidades autónomas españolas pertenecientes al proyecto de investigación PROGENSA® (Muestras control). De estas muestras, cuatro individuos se utilizaron para optimizar las reacciones de PCRs múltiplex (Muestras de prueba). Por otra parte, una familia de hermanos carnales de 60 hijos y sus reproductores (Muestras de validación) fueron utilizados para verificar la correcta segregación de los alelos y revalorar la facilidad de lectura del genotipado de cada marcador microsatélite mediante el *GremmProtocol* (Lee-Montero *et al.*, 2013).

Se extrajo ADN del tejido de la aleta caudal, utilizando el kit de DNAeasy (QIAGEN®) y luego se mantuvo a-20°C siguiendo las instrucciones proporcionadas por el fabricante. La integridad, cantidad y calidad del ADN se determinaron utilizando el espectrofotómetro *Nanodrop* 1000 v.3.7 (*Thermo Fisher Scientific*, Wilmington, Estados Unidos) y mediante electroforesis en gel de agarosa al 1% ($8V \cdot cm^{-1}$).

2.1.2 Marcadores microsatélites

De los microsatélites publicados en el mapa genético de la dorada (Franch *et al*., 2006), 138 parejas de cebadores se rediseñaron para que amplificaran bajo las mismas condiciones de PCR. A cada pareja de cebadores se le asignó un código interno para facilitar el trabajo de laboratorio. En la Tabla 1 se muestra el nombre de los loci, su código interno, su código de acceso en la base de datos GenBank, el fluorocromo asignado y la secuencia de los cebadores de cada marcador microsatélite usado en este trabajo. Cada marcador microsatélite fue probado mediante PCR simple utilizando las 16 muestras de ADN para confirmar su correcta amplificación, rango alélico, morfología, variabilidad genética y facilidad de lectura del genotipado.

La heterocigosidad observada (Ho), esperada (He) y el contenido de información polimórfica (PIC) de cada marcador, así como la probabilidad de exclusión combinada (CEP) de cada multiplex se estimaron usando el programa informático Cervus (v 3.0.3).

2.1.3 PCR simple

Las condiciones de PCR consistieron en una desnaturalización inicial a 95° C durante 10min, seguida de 28 ciclos de 94° C durante 30 segundos, 60° C durante 1minuto y 65° C durante 1minuto, con una extensión final de 60 º C durante 60 minutos. La reacciones se llevaron a cabo en un volumen final de 12,5µl con las siguientes concentraciones para cada componente: 1 X *GeneAmp PCR Buffer* II (100 mM Tris-HClpH 8,3, 500 mMKCl) (*Applied*

Biosystem®, USA), 3 mM $MgCl_2$, 0,2 mM de cada dNTP, 0,04 unidades μl^{-1} *AmpliTaq Gold* ADN polimerasa (*Applied Biosystem*®), 2,4-6,4ng•μl^{-1}de ADN y 0,2µM de cada cebador.

2.1.4 Evaluación de la facilidad de lectura del genotipado

La facilidad de lectura del genotipado de cada marcador microsatélite se evaluó mediante la identificación de los errores o potenciales errores de lectura y asignando a cada marcador una puntuación entre 1 y 3 según describieron Lee-Montero *et al*. (2013) mediante el *GremmProtocol*. Según esta clasificación 1 denota genotipos ambiguos en más del 30% de las muestras; 2, genotipos ambiguos en un 30% o menos de las muestras; y 3, genotipos sin ambigüedades en el 100% de las muestras.

2.1.5 PCRs múltiplex

Después de la evaluación de los marcadores microsatélites, aquéllos que no amplificaron o fueron monomórficos se desecharon. Con el resto de marcadores, se diseñaron 13 PCRs múltiplex que se denominaron ReM*sa* 1- 13 (*Redesigned multiplex Sparus aurata*). Los marcadores fueron combinados en cada múltiplex según su fluorocromo, su grupo de ligamiento y el rango del tamaño alélico. Aquellos microsatélites que no amplificaron en una múltiplex, se incluían en otra con el fin de maximizar el número de marcadores por múltiplex. Inicialmente, cada cebador fue utilizado con una concentración de 0,2µM en la PCR multiplex, modificando dicha concentración hasta conseguir una altura del pico entre 600 y 3000 RFU (*Relative Fluorescente Units*) para cada marcador microsatélite como describieron Navarro *et al*. (2008). Para la puesta a punto de cada PCR múltiplex se utilizaron las muestras

de prueba. Las condiciones PCR fueron las mismas que las utilizadas en la PCR simple inicial. Para comprobar la adecuada amplificación de los productos de PCR múltiplex se realizó una electroforesis cargando una alícuota del producto de PCR en un gel de agarosa al 2% durante 30 minutos ($8v \cdot cm^{-1}$). Tras comprobar su correcta amplificación1 µl de la reacción (diluido al 75% con agua Milli-Q) se mezcló con 9,75µl de Hi-Di formamida y 0,25µl del marcador de peso molecular *Gene Scan* LIZ 500 (*Applied Biosystem*®) y se cargó en un secuenciador automático ABI *Prism*® 3130XL (*Applied Biosystem*®). Los electroferogramas resultantes se analizaron usando el programa informático *GeneMapper* (v.3.7) (*Applied Biosystem*®).

2.2 Análisis de segregación de deformidades esqueléticas en dorada (*Sparus aurata* L.); ausencia de opérculo, lordosis, fusión de vértebras y complejo LSK

2.2.1 Experimento 1 (puesta masal con criba)

Se obtuvo un lote de huevos mediante puesta masal de un stock industrial de reproductores. Dicho stock estaba compuesto por 66 reproductores no deformes, y la proporción de sexos fue 1♀: 2♂.

Los huevos y las larvas se cultivaron en las instalaciones del Parque Científico Tecnológico Marino de la Universidad de Las Palmas de Gran Canaria (PCTM-ULPGC, Gran Canaria, España) y bajo las condiciones descritas por Roo *et al*.(2009). Cuando los alevines contaban con 111 días de edad (1,9 ± 0,02 g) (promedio ± error estándar), fueron cribaros y aquellos que presentaron algún tipo de deformidad, se retiraron del lote, con la excepción de

los individuos que presentaron complejo LSK (lordosis/escoliosis/cifosis) que se seleccionaron y se cultivaron separadamente en un tanque de fibra de vidrio de 1000 L. El porcentaje de individuos LSK encontrado en todo el lote fue 0,22%. Con respecto a los individuos considerados como normales, se transportaron a las instalaciones de la empresa Playa de Vargas 2001 S.L. (PLV2001, Gran Canaria, España) cuando contaban con 130 días de vida (4,8 ± 0,1 g). Estos peces fueron criados en condiciones industriales en una jaula y alimentado con pienso comercial de peces. El período de crecimiento duró hasta 509 días cuando los peces alcanzaron 419,7 ± 3,1 g.

Al final del experimento, una muestra de 846 peces de PLV2001 se sacrificó y se analizó visualmente para determinar la presencia o ausencia de deformidades según AquaExcel-ATOL (AquaExcel Project, 2013; ATOL: 0000087). Dichas deformidades se evaluaron mediante observación directa del esqueleto axial, tras filetearlos.

Las relaciones familiares entre los reproductores y la descendencia (846 de PLV2001 y 54 peces LSK del PCTM-ULPGC) se determinaron por el método de exclusión utilizando el software VITASSING (v8.2.1) (Vandeputte *et al*., 2006), tras el análisis de ADN y caracterización genética con la PCR múltiplex SMsa1 (*SuperMultiplex Sparus aurata*) descrita por Lee-Montero *et al*. (2013).

2.2.2 Experimento 2 (puesta masal sin criba)

Se recogieron huevos en dos días consecutivos de una puesta masal de un lote industrial de reproductores del PCTM-ULPGC, en el marco del proyecto PROGENSA®

(Afonso *et al*., 2012). Este lote de reproductores estaba compuesto por 59 reproductores no deformes, con una proporción de 1♀: 1.81♂.

Los huevos y las larvas se cultivaron en las instalaciones de PCTM-ULPGC, tal como se describe en el Experimento 1. 179 días después de la eclosión (17,2 ± 0,2 g), los alevines se marcaron individualmente con *Passive Integrated Transponder* (PIT) (*Trovan Daimler-Benz*) siguiendo el protocolo descrito por Navarro *et al*. (2006) y se transportaron a una jaula de la empresa CANEXMAR S.L. (Gran Canaria, España). Se criaron bajo condiciones intensivas y se alimentaron con alimento comercial. Ningún proceso de criba se llevó a cabo durante el cultivo de los peces, el cual se prolongó hasta 689 días, cuando los peces pesaron 524,4 ± 12,6 g.

Se realizó una evaluación visual inicial en el momento del marcaje (179 días), para determinar la presencia o ausencia de deformidades (ATOL: 0000087). Al final del experimento (689 días), una muestra de 810 peces se sacrificaron y se analizaron visualmente (análisis visual final), para determinar la presencia o ausencia de deformidades (ATOL: 0000087). Al igual que en el Experimento 1, se evaluaron las deformidades vertebrales directamente después del fileteado del pescado.

Las relaciones familiares entre los reproductores y la descendencia, se determinaron como se describe en el Experimento 1.

2.2.3 Experimento 3 (cruces dirigidos)

En las instalaciones del PCTM-ULPGC se establecieron diferentes cruces dirigidos con reproductores de dorada, más concretamente, 15 machos deformes y 10 hembras normales (N). Los reproductores deformes se clasificaron en tres grupos: ausencia de opérculo (O), lordosis (L) y fusión de vértebra (FV) según la deformidad que presentaban. Se constituyeron tres apareamientos (réplicas) por cada deformidad: NxO, NxL y NxFV. La proporción de sexos en cada tanque fue una hembra normal con dos machos deformes (1♀N: 2♂D). Además, una puesta masal con reproductores no deformes (1♀N: 2♂N) fue utilizada para conformar un cruce control (C). Los huevos de cada apareamiento se cultivaron cada uno separadamente, en las instalaciones de PCTM-ULPGC, tal como se describe en el Experimento 1. Los alevines de cada apareamiento se criaron por separado en tanques de fibra de vidrio de 1000 L hasta los 129 días (9,6 ± 0,1 g de peso).

Al final del experimento, se analizaron visualmente todos los peces procedentes de los cuatro cruces (11.503 individuos) para determinar la presencia o ausencia de deformidades esqueléticas (ATOL: 0000087).

2.2.4 Análisis estadístico

La asociación entre cualquier factor (reproductor, familia, cruce o experimento) y deformidad, se analizó con un modelo *log*lineal utilizando el programa estadístico SPSS (v18 *PASW Statistics*). El modelo *log*lineal da el significado de cualquier factor de deformidad (su prevalencia [i]) contra cualquier factor biológico o funcional (dependiendo de los datos [j]), organizado en una tabla de contingencia bidireccional, a través de los valores normalizados o

valores Z. Los valores Z >1,96 ó Z<-1,96indican un exceso o defecto significativo de la deformidad con respecto a la familia, reproductor, cruce o experimento, respectivamente.

$$\ln f_{ij} = \mu + \alpha_i + \beta_j + \alpha\beta_{ij}$$

Donde $\ln f_{ij}$ es la frecuencia esperada de cada deformidad (i) en cada familia, reproductor, cruce o experimento (j) considerado (ij); μ es el valor medio de los logaritmos de las frecuencias esperadas, α_i es el efecto del factor de deformidad (i), β_j es el efecto de la familia, reproductor, cruce o experimento (j) y $\alpha\beta_{ij}$ es el efecto debido a la interacción de estos factores.

En los Experimentos 1 y 2 se analizó la relación entre cada deformidad y todas las familias, machos y hembras. En el Experimento 3, se analizó la relación entre cada deformidad y el cruce, las familias y los tanques.

2.3 Identificación de QTLs asociados a deformidades esqueléticas en dorada (Sparus aurata L.): ausencia de opérculo, anomalía mandibular, lordosis, fusión de vértebra y complejo LSK

2.3.1 Material biológico, caracteres analizados y asignación parental

Estudio 1

El diseño de los cruces de reproductores, las condiciones de cultivo, el análisis fenotípico y la asignación parental están descritos con detalle en Negrín-Báez *et al*. (2014b).

La descendencia de un lote industrial de reproductores se cultivó hasta la edad de 509 días (419.7 ± 3,1 g) (promedio ± error estándar). A los 111 días dicha descendencia se analizó para determinar la presencia de deformidades, siendo sacrificados todos los peces deformes a excepción de los individuos LSK. Éstos fueron seleccionados y cultivados por separado. Las relaciones familiares se determinaron mediante caracterización genética usando la PCR múltiplex SMsa1 (*SuperMultiplex Sparus aurata*) (Lee-Montero *et al*., 2013). Al final del experimento, se analizaron fenotípica genéticamente y un total de 900 peces. De las 89 familias de hermanos carnales que se formaron, sólo seis de ellas, procedentes de dos machos (♂1L y ♂2L) y cuatro hembras (♀1L, ♀2L, ♀3L, ♀5L y ♀6L), incluyeron el total de individuos LSK (Negrín-Báez *et al*., 2014b). Cinco de estas seis familias (FAM1-5) se seleccionaron para el análisis de QTLs. El total de individuos por familia y su fenotipo se muestran en la Tabla 8.

Estudio 2

El diseño de los cruces de reproductores, las condiciones de cultivo, el análisis fenotípico y la asignación parental están descritos con detalle en Negrín-Báez *et al*. (2014b). Se cultivó la descendencia de un lote industrial de reproductores perteneciente al programa de mejora PROGENSA® (Afonso *et al*., 2012) y se criaron durante 689 días (524,4 ± 12,6 g). Un total de 810 peces se analizaron fenotípica y genéticamente. La estructura familiar se determinó como se describe en el Estudio 1, obteniéndose 66 familias de hermanos carnales. Sólo la prevalencia de la ausencia de opérculo mostró una asociación estadísticamente

significativa con algún reproductor o alguna familia: un macho, una hembra y dos de las familias. El 48,94% de los individuos con ausencia de opérculo fueron descendientes de este macho (♂1_O) (Negrín-Báez *et al*., 2014b). De esta manera, todos los descendientes de este macho (seis familias de medios hermanos, FAM6-11) se seleccionaron para el análisis de QTLs. El total de individuos por familia y su fenotipo se muestran en la Tabla 8.

Estudio 3

Se estableció un cruce con dos reproductores deformes (lordosis). Estos reproductores, a su vez, procedían de una F1 establecida con un macho deforme y una hembra normal descrita más extensamente por Negrín-Báez *et al*. (2014b). La descendencia se cultivó en las instalaciones del PCTM-ULPGC en condiciones descritas por Roo *et al*. (2009). A los 120 días (3,74±1,24 g), los peces se sacrificaron y se analizaron visualmente para determinar la presencia de cualquier tipo de deformidad (análisis inicial). Se observaron tres tipos de deformidades: fusión de vértebras, lordosis y anomalías de mandíbula. Una muestra de 152 individuos (FAM12) se seleccionó para el análisis de QTLs. Se realizó un segundo análisis mediante rayos X (*Mod. Senographer-DHR, General electric´s*, USA) para confirmar las deformidades detectadas en el análisis inicial. La proporción real de peces deformes y todos los tipos de deformidades detectados se muestran en la Tabla 8.

Genotipado

La descendencia y los reproductores de los tres estudios se genotiparon utilizando las 13 múltiplex PCR (ReM*sa*1- ReM*sa* 13) descritas en Negrín-Báez *et al*. (2014a). Estas múltiplex

PCR incluyeron un total de 106 marcadores de microsatélites ubicados en el mapa genético de esta especie (Franch *et al*., 2006; Senger *et al*., 2006).

Análisis de QTL

Se utilizó un modelo de regresión lineal (LR) (Knott *et al*., 1996) para la detección de los QTLs en todos los estudios usando el programa *GridQTL* (http://www.gridqtl.org.uk) (Seaton *et al*., 2006). Debido a la estructura familiar, en los Estudios 1 y 2 se realizó un análisis de regresión de medios hermanos y de hermanos carnales, mientras que en el Estudio 3 solo fue posible el de hermanos carnales. En ambos análisis, se estimó el umbral de significación tanto a nivel de todo el genoma como a nivel de cada cromosoma mediante un método de *bootstrapping*en p = 0,05 y p = 0,01 con un test de permutación de 10.000 iteraciones (Churchill y Doerge *et al*., 1994). Con respecto al análisis a nivel de todo el cromosoma, el QTL se consideró sugerente cuando su significación estuvo entre el 5% y el 1% y significativo cuando fue inferior al 1%. Con respecto al análisis a nivel de todo el genoma, el QTL fue significativo cuando estuvo entre el 5% y el 1% y altamente significativo cuando fue inferior al 1% (Rodríguez-Ramilo *et al*., 2011; Vallejo *et al*., 2014). No se incluyó ningún factor fijo o covariable en el modelo. Los intervalos de confianza se calcularon por *bootstrapping* de las muestras 10.000 veces (Visscher *et al*., 1996).

El efecto del QTL en términos del porcentaje de varianza explicada por el QTL (PVE) se estimó según Knott *et al*. (1996), utilizando la diferencia entre los modelos completos y reducidos, como se explica a continuación:

$$PVE_{HS}\ (\%) = 4 \times ((\text{RMS red} - \text{RMS total}) / \text{RMS red}) \times 100$$

$$PVE_{FS}\ (\%) = 2 \times ((\text{RMS red} - \text{RMS total}) / \text{RMS red}) \times 100$$

Donde HS es el análisis de medios hermanos, FS es el análisis de hermanos carnales, RMS red son los cuadrados medios residuales del modelo reducido en el que se incluyen todos los efectos excepto el del QTL y el RMS total son los cuadrados medios residuales del modelo en el que todos los efectos, incluido el del QTL, están incluidos.

La magnitud del efecto del QTL según la PVE se estableció siguiendo la clasificación de Massault *et al*. (2011), como efecto pequeño cuando la PVE fue inferior al 5% y efecto grande cuando fue mayor que 10%, y se incluyó en la clasificación: efecto medio cuando la PVE estuvo entre 5 y 10 %, y efecto extremadamente grande cuando fue mayor de 35%.

Análisis de asociación genotípica

Se determinó la asociación entre el fenotipo (normal vs anormal) y el alelo segregado de cada microsatélite cercano a cada QTL significativo. Para ello se realizaron tablas de contingencia y tests de Chi-cuadrado de *Pearson*. Los alelos se codificaron para discriminar entre los que vienen del padre y los que vienen de la madre como: s1, s2, d1, d2 (s=*sire*; d=*dam*). La segregación alélica se realizó por madre y por padre separadamente. Para los tests de Chi-cuadrado de *Pearson* se utilizó el siguiente modelo:

$$x^2 = \sum_{i=1}^{I} \sum_{j=1}^{J} \frac{(o_{ij} - e_{ij})^2}{e_{ij}}$$

Donde *o* = frecuencia, *e* = la frecuencia esperada, *i* = el valor del alelo y *j* = la categoría de deformidad (normal vs deforme). Se consideró asociación significativa cuando $P \leq 0.05$.

También se determinó el efecto del QTL en términos de asociación entre el fenotipo (normal vs deforme) y los genotipos de los marcadores microsatélites cercanos a cada QTL significativo. Se realizó un análisis de varianza de una vía (ANOVA) sobre los valores fenotípicos de la progenie para cada familia utilizando los genotipos individuales (s1d1, s1d2, s2d1, s2d2). Cada ANOVA proporcionó un valor de R^2 corregido que midió la reducción de la varianza fenotípica de la deformidad debida al modelo apropiado, proporcionando de este modo la proporción de la varianza predecible a partir de los genotipos.

Ambos análisis se realizaron utilizando el programa estadístico SPSS (PASW Statistics v18).

3. RESULTADOS Y DISCUSIÓN

3.1 Diseño de una batería de 13 PCRs múltiplex de marcadores específicos como una herramienta para la detección de QTLs en dorada (*Sparus aurata* L.)

De los 138 marcadores microsatélite rediseñados, 10 no amplificaron individualmente y dos eran monomórficos. De los restantes marcadores, 108 se utilizaron para conformar las 13 PCRs múltiplex. El número de marcadores por múltiplex osciló entre 6 y 11 y la concentración final de cada cebadores osciló entre 0,03 y 0,15 µM. El número final de marcadores de cada PCR multiplex y el código interno, fluorocromo, grupo de ligamiento, motivo de repetición nucleotídico, número de alelos, rango alélico, concentración de los cebadores, heterocigocidad observada (Ho) y heterocigocidad esperada (He), contenido de información polimórfica (PIC) y evaluación de la facilidad de lectura mediante *GremmProtocol* de todos sus marcadores se muestran en la Tabla 2.

Los valores de variabilidad observados fueron altos y similares a los encontrados para otras PCRs múltiplex para dorada (Navarro *et al*., 2008). Sin embargo, el tamaño de los productos de PCR en este trabajo fueron más cortos que los utilizados en otros estudios (de 51 a 205). Esto supone una ventaja, ya que cuando se utilizan muchos marcadores en una múltiplex, sus tamaños deben ser similares para evitar diferencias en la amplificación debido a las competencias de los cebadores. Por otro lado, los microsatélites de este estudio se evaluaron según su facilidad de lectura de genotipado y este es un parámetro crítico tan importante como lo es el polimorfismo (Lee-Montero *et al*., 2013). En la Tabla 3 se muestra

el número final de *loci* y la variabilidad genética para cada PCR múltiplex desarrolla en este trabajo.

La longitud del mapa de ligamiento genético de dorada es 1241,9 cM y presenta 26 GL (Franch *et al*., 2006). Para una eficiente detección de QTLs, es necesario un mapa de ligamiento genético con un nivel alto de saturación de marcadores moleculares. Las 13 PCRs múltiplex desarrolladas en este estudio cubren el 100% de los GL del mapa genético dorada, constituyendo así una herramienta potente y de bajo costo para la detección de QTLs. Además, los 18 marcadores no incluidos en las PCRs múltiplex finales, proporcionan a los genetistas la posibilidad de incluirlos posteriormente en un mapeo más exhaustivo, dado que sus correspondientes secuencias fueron rediseñadas para ser amplificadas en las mismas condiciones PCR.

3.2 Análisis de segregación de deformidades esqueléticas en dorada (*Sparus aurata* L.); ausencia de opérculo, lordosis, fusión de vértebras y complejo LSK

La prevalencia total de las deformidades (%) de la descendencia de los reproductores en los tres experimentos y el número de descendientes en cada experimento se muestra en la Tabla 4.

3.2.1 Experimento 1 (puesta masal con criba)

Del total de peces LSK que fueron seleccionados, sólo 54 sobrevivieron hasta el final del experimento (75% de mortalidad). Se obtuvo un 100% de éxito en la asignación parental, es decir, toda la descendencia (900 individuos) se asignó a sus correspondientes padre y madre

que sumaron un total de 28 reproductores (17 hembras y 11 machos). Se formaron un total de 89 familias de hermanos carnales, y sólo seis de ellos incluyeron el total de individuos LSK (54). Estas familias (llamadas msF1-msF6) estuvieron compuestas por seis reproductores: cuatro hembras y dos machos (llamados ms♀1-ms♀4 y ms♂1-ms♂2). La prevalencia de deformidades (%) (con sus valores Z) en los descendientes de estos reproductores y familias, con respecto al resto de reproductores (valor medio de la prevalencia), y con respecto a cada macho, hembra y familia, se muestran en la Tabla 5. Estos dos machos, tres de las hembras y cinco de las familias mostraron una relación estadísticamente significativa con la deformidad debido a un exceso en de individuos LSK ($Z_{family,\ LSK} > +2$; $P<0,05$). Es destacable que ms♂1 y ms♂2 fueron los responsables del 75% de los peces con FV, mientras ms♀2 fue responsable del 51.85% de los individuos LSK y el 25% de los peces con ausencia de opérculo. Las hembras no mostraron ningún descendiente con FV.

El complejo LSK fue descrito por primera vez en dorada como una triple deformidad de columna consistente en una repetición consecutiva de lordosis/escoliosis/cifosis desde la cabeza hasta la cola (Afonso *et al*., 2000). Estos autores diseñaron un esquema de cruzamiento que incluyó el cultivo por separado de 31 familias de hermanos carnales, y encontraron una interacción estadística significativa entre la prevalencia de esta deformidad y una familia, independientemente de las réplicas. En ese estudio, los individuos con deformidad complejo LSK representaron 0,2% de la descendencia; de manera similar a lo que se obtuvo en el presente estudio: 0,22% del total de la descendencia.

Al comparar la prevalencia de deformidades en los todos experimentos (Tabla 4), puede observarse que la deformidad complejo LSK presenta la prevalencia más baja en toda la descendencia (0-0.2%), revelando que es una deformidad extremadamente rara. Eso podría ser la razón por la cual ha sido incluido rara vez en los estudios de deformidades (Afonso *et al*., 2000; Ebrahimnezhad *et al*., 2009).

3.2.2 Experimento 2 (puesta masal sin criba)

Se obtuvo el 100% de éxito en la asignación parental, es decir, todos los descendientes (810 individuos) fueron asignados a sus correspondientes reproductores que sumaron 45 (19 hembras y 26 machos) y formaron 66 familias de hermanos carnales. En el análisis inicial, a los 179 días después de la eclosión, el 7% de descendencia mostró ausencia de opérculo, y no se observaron individuos con lordosis ni con FV. La prevalencia de ausencia de opérculo fue independiente de la familia y del reproductor ($-1{,}96 < _{\text{reproductor/familia,deformidad}} < +1{,}96$; $P > 0{,}05$). En el análisis final, a los 689 días, un 7,65% de los peces mostró alguna deformidad. Sólo la prevalencia de ausencia de opérculo mostró una asociación significativamente estadística ($Z > 2$, $P < 0{,}05$) con algún reproductor y con las familias formadas por éstos. El 48,94 % de los individuos con ausencia de opérculo, fueron descendiente de un solo macho (llamado mw♂1), siendo esta asociación estadísticamente significativa. Sólo dos familias, la formada por mw♂1 y dos hembras (mw♀1 y mw♀3) con el mw♂1, mostraron una prevalencia significativa con la ausencia de opérculo, representando el 12,77 y 21,28 % de todos los individuos,

respectivamente. La prevalencia de las deformidades, dada en porcentajes, en la descendencia de mw♂1 en el análisis final se muestra en la Tabla 6.

El análisis inicial de este experimento no mostró ninguna relación entre la prevalencia de la ausencia de opérculo y cualquiera de las familias o los reproductores. Por el contrario, a los 539 días, sí se observó relación significativa entre dos de los reproductores (mw♂1 y mw♀1) y dos de las familias (mwF1 y mwF3) y la prevalencia de la ausencia de opérculo en sus descendientes. Lee-Montero *et al*. (2014) también indicaron una un aumento hasta valores medios (0,11) de la heredabilidad para esta deformidad en dorada a talla comercial (300-800 g) con respecto a talla más pequeña (15 g). Además, estos autores, al marcar individualmente todos los individuos, pudieron demostrar que algunos peces regeneran el opérculo a lo largo de su desarrollo apoyando la hipótesis propuesta por otros autores (De Wolf *et al*., 2004; Verhaegen *et al*., 2007; Beraldo y Canavese, 2011). En este trabajo, tampoco se observó asociación familiar a talla pequeña, mientras que si se encontró a talla mayor. Todos estos resultados sugieren que la prevalencia de la ausencia de opérculo a talla pequeña está influenciada principalmente por factores medioambientales (Koumoundouros *et al*., 1997; Beraldo *et al*., 2003; Roo *et al*., 2005; revisado por Boglione *et al*., 2013), mientras que su determinación genética se aprecia más significativamente a tallas más grandes.

3.2.3 Experimento 3 (cruces dirigidos)

De las tres réplicas de cada tipo de cruce, los siguientes fueron viables: los tres NxO, dos NxL y sólo uno NxVF. Una alta mortalidad fue observada en un cruce de NxO y en un cruce NxL, por lo que la descendencia completa no pudo ser analizada. De los 11.503 analizaron peces, el 6,15% mostraron alguna de las deformidades analizadas. La prevalencia (%) (con sus valores de Z) de cada tipo de deformidad en la descendencia de los diferentes cruces se muestra en la Tabla 7. Como se puede observar, el tipo de deformidad estuvo fuertemente asociado con el tipo de cruce: el cruce NxO mostró un exceso de ausencia de opérculo, el cruce NxL mostró un exceso de ausencia de opérculo y lordosis, y el cruce de NxVF mostró un exceso de FV. De la misma forma, el cruce control mostró un exceso de individuos normales ($Z_{cruce,\ deformidad}$>+5; P<0,01). El efecto familiar (medios hermanos), que sólo pudo ser analizado en dos réplicas del cruce NxO, fue estadísticamente significativo. Una familia demostró un exceso de individuos con ausencia de opérculo (9,98%) y un defecto de individuos normales (89,54%), contrariamente a lo observado en la otra familia (0,98% y 99,69%, respectivamente) ($Z_{family,deformity}$> +3,6; P<0,01).

En los cruces NxO y NxL, se observó una prevalencia estadísticamente significativa con la ausencia de opérculo. Además, se observó que una familia fue responsable de la alta prevalencia de la deformidad del opérculo en este cruce. Estos resultados observados para ausencia de opérculo en este experimento, coinciden con los obtenidos en el Experimento 2 y los obtenidos por Lee-Montero *et al*. (2014).

En este experimento, la prevalencia de las deformidades en los descendientes de los reproductores deformes fue significativamente mayor que en la descendencia del cruce de control y que en Experimento 2 a tamaño inicial. De hecho, en el Experimento 3, se detectó la presencia de deformidades de columna fácilmente incluso a una edad temprana, posiblemente debido a una mayor intensidad o severidad de la deformidad (Bardon *et al*., 2009). Por el contrario, en los Experimentos 1 y 2, los individuos deformes no fueron detectados hasta que alcanzaron una talla mayor.

3.3 Identificación de QTLs asociados a deformidades esqueléticas en dorada (Sparus aurata L.): ausencia de opérculo, anomalía mandibular, lordosis, fusión de vértebra y complejo LSK

3.3.1 Mapa genético

El desarrollo de las nuevas tecnologías genéticas ha aumentado significativamente en las últimas décadas. En el presente trabajo, se utilizó un set de 13 multiplex PCR descrito para esta especie para genotipar con 106 microsatélites todas las familias. El uso de PCR multiplex permite una automatización en el genotipado lo que supone una reducción significativa de los costes y minimización de errores (Negrín-Báez *et al*., 2014a). El espacio promedio entre estos marcadores microsatélites fue de 14,6 cm, menor que la distancia máxima recomendada de 20 cm para la búsqueda de QTL (Massault *et al*., 2008). A pesar de que es una distancia más grande entre los marcadores que en otros estudios de QTLs para esta especie (Loukovitis *et al.*, 2011, 2012 y 2013; Boulton *et al.,* 2011), es de resaltar que en el presente trabajo se

cubrió un promedio de 72,6% (901,4 cm) de la longitud total del mapa de ligamiento genético de dorada (1241,9 cm). En esos otros estudios, sin embargo, la longitud total cubierta fue significativamente menor. El número de marcadores microsatellites analizados por familia, la longitudud de mapa cubierta, la distancia media entre marcadores y el número de marcadores por GL se muestran en la Tabla 9.

En acuicultura, los estudios de búsqueda de QTLs en familias han sido exitosos. En este tipo de método, el tamaño de la muestra, la heredabilidad del carácter, el efecto medio sustitución alélica de los alelos implicados y la asociación con marcadores cercanos determinan la eficacia de la detección de QTLs (Wang *et al*., 2006). En el presente trabajo, se utilizó el método de regresión lineal en familias de hermanos carnales y medios hermanos mediante el programa informático *GridQTL*, que se ha evidenciado como robusto para su uso con caracteres discretos, incluyendo los binarios (Gorman *et al*., 2011), que es el caso de las deformidades. Se analizaron entre 1 a 6 familias, dependiendo de la población, con un número de individuo por familia de 5 a 152, y las heredabilidades para la presencia o ausencia de algunas de las deformidades esqueléticas estimadas para esta especie mostraron valores medio-bajos (Lee-Montero *et al*., 2014).

3.3.2 Estudio 1

El complejo LSK es una triple deformidad de la columna de fácil diagnóstico en edades tempranas y de baja prevalencia de alevines de dorada. Sin embargo, es muy grave y produce una elevada mortalidad, por lo que las empresas de cría realizan cribas para

detectarlos y eliminarlos antes de vender los lotes a las empresas de engorde, lo que implica pérdidas económicas (Negrín-Báez *et al*., 2014b).

En este estudio se realizó un análisis de regresión lineal para las familias de hermanos carnales FAM1-5, así como un análisis de regresión para las familias de medios hermanos a partir de los machos ♂1L y ♂2L (medios hermanos paternos) y a partir de las hembras ♀1L, ♀2L y ♀3L (medios hermanos maternos). Ambos análisis, además, se llevaron a cabo a nivel de genoma y cromosoma. En este estudio, los QTLs que resultaron significativos en al menos dos de los análisis relacionados con la estructura familiar fueron considerados sólidos. Se detectó un total de 14 QTLs para la deformidad de complejo LSK. El tipo de análisis, la localización de cada QTL detectado, los intervalos de confianza (cM) y los valores de significación estadística (F) se muestran en la Tabla 10. Este alto número de QTLs sugiere que esta deformidad está influenciada por una interacción compleja entre varias regiones del genoma, lo que era de esperar ya que es una deformidad a su vez compuesta por otras tres. Esto está también en concordancia con el origen genético previamente propuesto para esta deformidad por Afonso *et al*. (2000) y Negrín-Báez *et al*. (2014b).

Cuatro de los QTLs detectados (QTLSK2, 5, 10 y 12) fueron considerados como sólidos, siendo además significativos a nivel de genoma en, al menos, un análisis, lo que es de resaltar ya que es el nivel estadístico más riguroso (Loukovitis *et al*., 2012). El porcentaje de variación fenotípica explicada por los QTLs considerados sólidos, mostraron unos efectos extremadamente grandes en términos de PVE (Tabla 13). Estos valores pudieron estar algo sobreestimados, lo cual es común en los estudios de QTLs (Wang *et al*., 2006), posiblemente

debido al tipo de análisis (regresión lineal frente a modelos mixtos) (Rowe *et al*., 2006) y por el efecto Beavis (Xu, 2003). En cualquier caso, está claro que el efecto de los cuatro QTL sólidos es extremadamente grande, especialmente el QTLSK10 y el QTLSK12, de hecho, son los únicos QTL descritos en dorada que han mostrado un efecto extremadamente grande (Massault *et al*., 2010; Boulton *et al.,* 2011; Loukovitis *et al.,* 2011, 2012, 2013). Estos resultados indican que la deformidad LSK, podría estar determinada principalmente por estos pocos QTLs con gran efecto. Estos sólidos QTLs se han encontrado analizando solo 78, aunque no es de extrañar teniendo en cuenta estas 5 familias proceden de un estudio previo en el que sólo seis familias de 89 mostraron la incidencia total de individuos LSK, obteniéndose una relación significativa entre los reproductores y la familia con esta deformidad (Negrín-Báez *et al*., 2014b).

Además, se analizaron todos los marcadores microsatélites localizados en un GL donde se detectó un QTL sólido para el complejo LSK. Los marcadores microsatélites analizados cercanos a cada sólido QTL, la asociación significativa de los alelos de cada reproductor y el porcentaje de varianza fenotípica explicada por el genotipo (R^2) se muestran en la Tabla 13. Los marcadores *BId-39-T* (28,9 cM) y *P96* (40,1 cM) fueron los marcadores más cercanos para el QTLSK2 y mostraron una asociación significativa entre la segregación alélica de una hembra y los descendientes con complejo LSK. Todos estos descendientes deformes heredaron el alelo 74 para el marcador *BId-39-T* y el alelo 78 para el marcador *P96*, mientras que ningún individuo normal mostró esos alelos. También se observaron genotipos del marcador *BId-39-T* que solo presentaban los individuos LSK en 3 de las 5 familias y

genotipos del *P96* en 1 una de las familias. En el QTLSK5 el marcador *Did-22-F* (37.7 cM) mostró una asociación significativa entre la segregación alélica de una hembra y los descendientes con complejo LSK. Todos los descendientes deformes con complejo LSK de 2 de las 5 familias presentaban un genotipo diferente de los individuos normales. Sólo un marcador microsatélite estuvo localizado cerca del QTLSK10, el marcador *Gt57* (0 cm). El test Chi-cuadrado de *Pearson* (X^2) reveló una relación significativa entre la segregación alélica de un macho y una hembra y su descendientes con complejo LSK ($P<5.10^{-4}$y $P\leq4.10^{-3}$, respectivamente). En 3 de las 5 familias, todos los individuos LSK presentaban un genotipo que lo tenían los no deformes. Los marcadores microsatélites más cercanos para QTLSK12 fueron el *DId-03-T* y el *Bt-14-F* (1,3 cM). Para el marcador *DId-03-T*, el análisis de tablas de contingencia y el test Chi-cuadrado de *Pearson* X^2 mostró una alta y significativa asociación entre la segregación alélica de los reproductores y los descendientes con complejo LSK, tanto para los machos ($P\leq4.10^{-5}$) como para las hembras ($P\leq0,01$). Todos los individuos con esta deformidad heredaron el alelo 126, mientras que ningún individuo normal lo hizo, lo mismo se observó a nivel de genotipo en 4 de las 5 familias. El marcador *Bt-14-F*, también mostró una elevada asociación significativa entre la segregación alélica de los machos ($P\leq0,01$) y de las hembras ($P<5.10^{-3}$). En este caso, todos los descendientes mostraron el alelo 110 para este marcador, el cual no aparece en los individuos normales y las 5 familias mostraron genotipos distintos para los individuos con complejo LSK que para los individuos no deformes.

Este fuerte vínculo entre estos QTL sólidos y sus marcadores son resultados muy importantes, como la relación entre el marcador *ID13* y un QTL para la resistencia a la

pasteurelosis encontrado por Massault *et al*. (2010). Por otro lado, en tres de estos QTL sólidos (QTLSK2, QTLSK5 y QTLSK10), los intervalos de confianza del 95% fueron grandes (entre 20 y 47 pb), lo que podría tener un efecto negativo en una selección MAS. Esto se puede resolver en el futuro aumentando el tamaño de la familia, usando más microsatélites para reducir el espacio entre los marcadores o usando nuevos marcadores (SNPs) (Loukovitis *et al*., 2012). Sin embargo, el QTLSK12, mostró un intervalo de confianza del 95% pequeño (4 ó 10 pb según el análisis), lo que unido a la fuerte vinculación de este QTL a sus marcadores, indica la importancia de incluir este QTL en un programa de selección MAS. En este sentido, en un programa de mejora genética en dorada, en condiciones industriales, es necesario el uso de marcadores moleculares para determinar el pedigrí (Navarro *et al*., 2008, 2009a, 2009b). El marcador *Bt-14-F* (QTLSK12) está incluido en la multiplex PCR SMsa1 que fue propuesta como un panel de referencia de esta especie (Lee-Montero *et al*., 2013).

Por lo tanto, los resultados obtenidos en este estudio confirman el origen genético de esta deformidad esquelética severa y muestran cuatro QTLs sólidos, dos de ellos (QTLSK10 y QTLSK12) fuertemente vinculados con la deformidad del complejo LKS; así como cinco marcadores moleculares (*Bid-39-T*, *P96*, *GT57*, *Did-03-T* y *Bt-14-F*) como posibles candidatos a ser incluidos en una selección MAS para esta especie, especialmente *Bt-14-F* (QTLSK12). Por otra parte, la selección MAS de los reproductores apropiados contra la deformidad del complejo LSK disminuiría la prevalencia de esta deformidad en empresas de cría y, al mismo tiempo, también podría disminuir la prevalencia de otras deformidades esquelética (lordosis y fusión de vértebras), ya que los resultados de un estudio previo de

segregación sugirió una posible relación entre estas deformidades (Negrín-Báez *et al*., 2014b), aumentando aún más las ganancias de las empresas.

Sin embargo, para aumentar la precisión de la posición de los QTLs detectados y sus efectos, sería recomendable utilizar más marcadores microsatélites y familias más grandes para las regiones de interés, en futuros estudios.

3.3.3 Estudio 2

La falta de opérculo, la anomalía externa más común en dorada, reduce la calidad del pescado, disminuyendo su valor de mercado (Beraldo *et al*., 2003). Esta deformidad es fácilmente detectable en edades tempranas, ya que es evidente, y se puede observar a partir de los 25 días de larva (revisado por Boglione *et al*., 2013). Muchos estudios que han demostrado que es posible reducir la prevalencia de esta deformidad en alevines mediante el control de los factores ambientales (Roo *et al*., 2005; Beraldo *et al*., 2003; Prestinicola *et al*., 2013), pero no su eliminación. Por lo tanto, las empresas de cría realizan cribas manuales. A pesar de esta estrategia, se encuentran peces con esta deformidad a talla comercial. Esto se debe a que esta deformidad puede aparecer en peces hasta 269 días de edad (Lee-Montero *et al*., 2014). También hay estudios que han sugerido que mientras que la prevalencia de la falta de opérculo en peces pequeños está influenciada principalmente por el medio ambiente, a edades mayores presenta una determinación genética significativa (Lee-Montero *et al.*, 2014; Negrín-Báez *et al*., 2014b). Por todas estas razones, la selección MAS podría ser una estrategia muy útil para minimizar aún más la prevalencia de esta deformidad durante todo su

desarrollo. Los resultados del presente estudio revelaron la presencia de dos QTLs significativos en el GL9 (QTLOP1) y en el GL10 (QTLOP2); y dos QTLs sugerentes en el GL12 (QTLOP3) y GL14 (QTLOP4) relacionados con la esta deformidad (Tabla 11). El análisis de regresión lineal para las familias de medios hermanos del macho ♂1$_O$ fue el análisis más robusto debido al diseño de la estructura familiar. Los QTLs que presentaron significancia en este análisis, se consideraron QTLs sólidos. Estos QTLs explican alrededor del 25% de la variación fenotípica de esta deformidad, según sus valores PVE (Tabla 13). Esto supone que estos QTL tienen un gran efecto, que es suficiente para iniciar la aplicación de MAS en programas de mejora (Yue, 2014). Sin embargo, sus intervalos de confianza del 95% fueron muy grandes, por lo que sería necesario establecer con mayor exactitud las posiciones de estos QTLs, utilizando nuevos marcadores microsatélites. De hecho, hay más marcadores microsatélites (7 en LG9 y 3 en LG10) en el mapa de ligamiento genético de la dorada (Franch *et al.*, 2006; Massault *et al.*, 2010), que no se incluyeron en el set de multiplex y podrían ser utilizados.

Respecto a la asociación entre los genotipos y esta deformidad, los marcadores microsatellites cercanos a los QTLOP1 y QTLOP2, la asociación significativa de los alelos de cada reproductor y el porcentaje de varianza fenotípica explicada por el genotipo (R^2) se muestran en la Tabla 13. Para el QTLOP1 los marcadores analizados fueron el *CId-89-H* (26,7 cM) y el *Gd-78-F* (40,5 cM), sin embargo el *CId-89-H* resultó ser un marcador no informativo, ya que los reproductores resultaron homocigóticos para este *locus*. El marcador *GD-78-F* mostró una valor significativo para el Chi-cuadrado de *Pearson*, X^2, (valor de P =

0,02) de la segregación alélica paterna. No se encontró ninguna asociación significativa para los marcadores cercanos al QTLOP2.

3.3.4 Estudio 3

El presente estudio proporciona por primera vez, siete QTLs para tres de las deformidades esqueléticas más relevantes y frecuentes a partir del análisis de una familia de hermanos completos de dorada (Tabla 12). Los marcadores microsatélites cercanos analizados, la asociación significativa de los alelos de cada reproductor y el porcentaje de varianza fenotípica explicada por el genotipo (R^2) se muestran en la Tabla 13.

La lordosis y la fusión vertebral son las deformidades esqueléticas más importantes, ya que además de que reducen el peso de los peces, reducen fuertemente su valor comercial (Afonso *et al*., 2000; revisado por Boglione *et al*., 2013). Su prevalencia se asocia a condiciones ambientales (revisado por Boglione *et al.*, 2013), pero también se ha propuesto un componente genético (Lee-Montero *et al.*, 2014; Negrín-Báez *et al*., 2014b.). Incluso un QTL significativo para la escoliosis se ha descrito en guppy (*Poecilia reticulata*), el cual contiene el *MTNR1B*, un gen candidato para la escoliosis idiopática humana (Gorman *et al*., 2011). Para la fusión vertebral, de los tres QTLs detectados en este estudio, uno fue significativa (QTLFV3) y se localizó en LG21. Su efecto fue pequeño, de acuerdo a su PVE, sin embargo, se encuentra en un corto LG, por lo que la asociación entre los marcadores y el QTL podría ser más fuerte (Massault *et al*., 2010). De esta manera, el marcador *Hd-46-T* mostró una asociación significativa de la segregación alélica del padre ($P \leq 0,03$) con esta

deformidad en los descendientes, y además su genotipo explicó el 3,6% de la variación fenotípica. Sin embargo el marcador, también cercano, *DId-12-F* fue no informativo, ya que los reproductores fueron homocigotos para este *locus*.

De los dos QTLs encontrados para lordosis, uno fue significativo (QTLORD1) y se encontró en el LG9. Es de resaltar que este QTL también fue significativo a nivel del genoma, que es un nivel estadístico más riguroso (Loukovitis *et al*., 2012). Sin embargo, su efecto, en términos de PVE, también fue pequeño y no se encontró asociación significativa entre los genotipos de ninguno de los marcadores de su LG y la deformidad. Los marcadores microsatélites más cercanos fueron el *Gd-78-F* (40,5 cM) y el *Cld-89-H* (26,7 cM), pero fueron no informativos (al igual que lo explicado arriba). No obstante, como el LG es el mismo del QTL sólido para la falta de opérculo (QTLOP1), si se realizara un mapeo más fino en este cromosoma en una nueva población, se podría medir y analizar también la lordosis. Este QTL y el de la fusión vertebral, confirmaron el origen genético de estas deformidades espinales.

La deformidad de mandíbula es también una deformidad esquelética frecuente, aunque con menor impacto económico. Una gran cantidad de estudios relacionan la prevalencia de esta deformidad con las condiciones ambientales (revisado por Boglione *et al*., 2013), pero no con factores genéticos. De hecho, Lee-Montero *et al*. (2014) estimaron una heredabilidad no distinta de cero para deformidades de cabeza. Esto podría ser explicado porque en las deformidades de cabeza, además de incluir las anomalías mandibulares, incluyeron otros tipos de deformidades de cabeza más difíciles de evaluar que pudieron aumentar la varianza

ambiental. En contraste, en este estudio se encontró un QTL significativo para la deformidad de la mandíbula localizado en el LG 13 (QTLSJW1), así como otro sugerente. Según el PVE, el efecto de QTLSJW1 también fue pequeño, sin embargo, una asociación significativa entre la segregación alélica de madre y la deformidad de mandíbula se detectó en los dos marcadores cercanos, que fueron el *Cid-26-H* (27,9 cM) y el *Cid-03-F* (30 cM). El genotipo de ambos marcadores explicaron el 11,4 y el 7,8% de la varianza fenotípica (R^2). Esto sugiere que aunque este QTL mostró un efecto pequeño, podría estar cerca de los marcadores analizados. De hecho, los dos marcadores están separados 2,1 pb de acuerdo con el mapa genético de dorada (Franch *et al*., 2006). Este QTL, demuestra por primera vez para la dorada, que la deformidad de mandíbula está determinada genéticamente, además de estar influenciada por factores ambientales.

Por otra parte, los tres QTLs significativos de este estudio (QTLFV3, QTLORD1, QTLSJW1) se detectaron usando una única familia de hermanos carnales. Esto proporciona una gran limitación, por lo que los QTLs podrían estar segregando en ambos reproductores y pueden no ser representativos de otras familias de dorada (Wang *et al.*, 2006; Ruan *et al.*, 2010). Por otra parte, sus efectos fueron pequeños y, aunque también los intervalos de confianza eran pequeños, este fue posiblemente debido precisamente a que el efecto fue pequeño.

Los resultados del presente estudio muestran los primeros QTL para lordosis, la fusión vertebral y deformidades de la mandíbula en dorada que en un futuro podrían ayudar a reducir la prevalencia de estas deformidades mediante selección MAS y resolver uno de los

problemas más importantes en la acuicultura. Sin embargo, los QTLs detectados deberían ser confirmados en otras familias de dorada antes de utilizar su información.

4. CONCLUSIONES

1. Se ha desarrollado una batería de 13 múltiplex PCRs, llamadas ReM*sa*1 a ReM*sa*13, que incluyen 106 marcadores microsatélires del mapa genético de dorada que cubren el 100% de los grupos de ligamiento y que constituye una potente herramienta para la búsqueda de QTLs en esta especie.

2. Se han rediseñado y evaluado 20 marcadores microsatélites adicionales, que amplifican en las mismas condiciones que la batería ReM*sa* y que ofrecen a los investigadores la oportunidad de incluirlos en futuros análisis.

3. Se determinó que las deformidades esqueléticas en dorada: complejo LSK, lordosis y fusión de vértebras tienen un origen genético, ya que presentaron una asociación significativa con la estructura familiar.

4. La alta prevalencia de las deformidades lordosis y fusión de vértebras en la descendencia de los reproductores responsables del complejo LSK sugieren una relación entre estas tres deformidades en dorada.

5. La deformidad ausencia de opérculo en esta especie a talla pequeña está influenciada mayoritariamente por factores ambientales, mientras que a talla comercial es más evitente su determinación genética.

6. El control tanto genético como fenotípico de los reproductores de dorada en apareamientos dirigidos afecta a la calidad de la puesta en términos de viabilidad y prevalencia de deformidades.

7. Se han relacionado 14 QTLs con la deformidad complejo LSK en dorada, de los cuales cuatro (en los grupos de ligamiento 5, 8, 17 y 20) fueron considerados los más sólidos ya que mostraron una muy alta responsabilidad en la variabilidad fenotípica de esta deformidad.

8. El genotipo de cinco marcadores microsatélites cercanos a estos cuatro sólidos QTLs para el complejo LSK, mostró una fuerte asociación con la deformidad en la descendencia y se proponen como potenciales candidatos para la Selección Asistida por Marcadores en esta especie.

9. Los dos QTLs significativos detectados para la deformidad ausencia de opérculo en dorada (en los grupos de ligamiento 9 y 10) presentaron un efecto medio en la varianza fenotípica.

10. Una estructura familiar de hermanos carnales en dorada presentó tres QTLs, uno para cada una de las deformidades analizadas: lordosis en el grupo de ligamiento 9, fusión de vértebras en el 21 y anomalía mandibular en el 13. Siendo recomendable su confirmación en otras familias.

11. Por primera vez, para dorada, se demuestra el origen genético para la deformidad de mandíbula.

12. Estos resultados suponen un gran paso hacia la localización de los genes que determinan el desarrollo de las deformidades esqueléticas estudiadas en dorada, y ofrecen una herramienta a la industria de esta especie para disminuir la prevalencia de las deformidades dentro de un programa de mejora.