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Identification of the Major Sex-Determining Region of Turbot (*Scophthalmus maximus*)

Paulino Martínez,*,[1] Carmen Bouza,* Miguel Hermida,* Jesús Fernández,[†] Miguel Angel Toro,[‡] Manuel Vera,* Belén Pardo,* Adrián Millán,* Carlos Fernández,* Román Vilas,* Ana Viñas,* Laura Sánchez,* Alicia Felip,[§] Francesc Piferrer, Isabel Ferreiro[††] and Santiago Cabaleiro[‡‡]**

**Departamento de Xenética, Universidade de Santiago de Compostela, Facultade de Veterinaria, 27002 Lugo, Spain, [†]Departamento de Mejora Genética Animal, Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, 28040 Madrid, Spain, [‡]Departamento de Producción Animal, ETS Ingenieros Agrónomos, Ciudad Universitaria, 28040 Madrid, Spain, [§]Instituto de Acuicultura de Torre de la Sal, Consejo Superior de Investigaciones Científicas, Ribera de Cabanes, 12595 Castellón, Spain, **Institut de Ciències del Mar, Centro Superior de Investigaciones Científicas, 08003 Barcelona, Spain, [††]Departamento I+D Stolt Sea Farm S.A, Lira, 15292 Carnota, A Coruña, Spain and [‡‡]Cluster de la Acuicultura de Galicia, Punta de Couso s/n, 15965 Aguiño (Ribeira), A Coruña, Spain*

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ABSTRACT

Sex determination in fish is a labile character in evolutionary terms. The sex-determining (SD) master gene can differ even between closely related fish species. This group is an interesting model for studying the evolution of the SD region and the gonadal differentiation pathway. The turbot (*Scophthalmus maximus*) is a flatfish of great commercial value, where a strong sexual dimorphism exists for growth rate. Following a QTL and marker association approach in five families and a natural population, we identified the main SD region of turbot at the proximal end of linkage group (LG) 5, close to the SmaUSC-E30 marker. The refined map of this region suggested that this marker would be 2.6 cM and 1.4 Mb from the putative SD gene. This region appeared mostly undifferentiated between males and females, and no relevant recombination frequency differences were detected between sexes. Comparative genomics of LG5 marker sequences against five model species showed no similarity of this chromosome to the sex chromosomes of medaka, stickleback, and fugu, but suggested a similarity to a sex-associated QTL from *Oreochromis* spp. The segregation analysis of the closest markers to the SD region demonstrated a ZW/ZZ model of sex determination in turbot. A small proportion of families did not fit perfectly with this model, which suggests that other minor genetic and/or environmental factors are involved in sex determination in this species.

SEX ratio is a central demographic parameter directly related to the reproductive potential of individuals and populations (Penman and Piferrer 2008). The phenotypic sex depends on the processes of both sex determination and sex differentiation. Exogenous factors, such as temperature, hormones, or social behavior, can modify the gonad development pathway in fish (Baroiller and D'Cotta 2001; Piferrer and Guiguen 2008). Both genetic (GSD) and environmental sex determination has been reported in this group (Devlin and Nagahama 2002; Penman and Piferrer 2008), although primary sex determination is genetic in most species (Valenzuela *et al.* 2003). Among GSD, single, multiple, or polygenic sex-determining (SD) gene systems have been documented (Kallman 1984; Matsuda *et al.* 2002; Lee *et al.* 2004; Vandeputte *et al.* 2007).

Sex determination in fish can evolve very rapidly (Woram *et al.* 2003; Peichel *et al.* 2004; Ross *et al.* 2009). Different sex determination mechanisms have been reported between congeneric species and even between populations of the same species (Almeida-Toledo and Foresti 2001; Lee *et al.* 2004; Mank *et al.* 2006). The evolution of sex chromosomes involves the suppression of recombination between homologous chromosomes probably to maintain sex-related coadapted gene blocks (Charlesworth *et al.* 2005; Tripathi *et al.* 2009). The sex determination pathway appears to be less conserved than other developmental processes (Penman and Piferrer 2008). However, differences are more related to the top of the hierarchy in the developmental pathway, while downstream genes are more conserved (Wilkins 1995; Marín and Baker 1998). As a consequence, the SD master gene in fish can vary among related species (Kondo *et al.* 2003; Tanaka *et al.* 2007; Alfaqih *et al.* 2009). In this sense, fish represent an

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[1]*Corresponding author:* Departamento de Xenética, Universidade de Santiago de Compostela, Facultade de Veterinaria, 27002 Lugo, Spain. E-mail: paulino.martinez@usc.es

attractive model for studying the evolution of SD mechanisms and sex chromosomes (Peichel *et al.* 2004; Kikuchi *et al.* 2007).

A low proportion of fish species have demonstrated sex-associated chromosome heteromorphisms (Almeida-Toledo and Foresti 2001; Devlin and Nagahama 2002; Penman and Piferrer 2008). This is congruent with the rapid evolution of the SD region in fish, and thus in most species the male and female version of this chromosome region appears largely undifferentiated. In spite of this, indirect clues related to progenies of sex/chromosome-manipulated individuals or to segregation of morphologic/molecular sex-associated markers indicate that mechanisms of sex determination in fish are similar to other vertebrates (Penman and Piferrer 2008). With the arrival of genomics, large amounts of different genetic markers and genomic information are available for scanning genomes to look for their association with sex determination. Quantitative trait loci (QTL) (Cnaani *et al.* 2004; Peichel *et al.* 2004) or marker association (Felip *et al.* 2005; Chen *et al.* 2007) approaches have been used to identify the SD regions in some fish species. Also, microarrays constructed from gonadal ESTs have been applied to detect differentially expressed genes in the process of gonadal differentiation (Baron *et al.* 2005). Further, the increased genomic resources in model and aquaculture species have allowed the development of both comparative genomics (Woram *et al.* 2003; Kikuchi *et al.* 2007; Tripathi *et al.* 2009) and candidate gene (Shirak *et al.* 2006; Alfaqih *et al.* 2009) strategies to identify and characterize the SD region in fish. This has permitted the identification of the SD region in eight fish, including both model and aquaculture species (reviewed in Penman and Piferrer 2008).

The turbot is a highly appreciated European aquaculture species, whose harvest is expected to increase from the current 9000 tons to >15,000 tons in 2012 (S. Cabaleiro, personal communication). Females of this species reach commercial size 4–6 months before males do, explaining the interest of the industry in obtaining all-female populations. Although some differences between families can be observed in the production process at farms, sex ratio is usually balanced at ~1:1. Neither mitotic nor meiotic chromosomes have shown sex-associated heteromorphisms in turbot (Bouza *et al.* 1994; Cuñado *et al.* 2001). The proportion of sexes observed in triploid and especially gynogenetic progenies moved Cal *et al.* (2006a,b) to suggest an XX/XY mechanism in turbot with some additional, either environmental or genetic, factor involved. However, Haffray *et al.* (2009) have recently claimed a ZZ/ZW mechanism on the basis of the analysis of a large number of progenies from steroid-treated parents. These authors also suggested some (albeit low) influence of temperature in distorting sex proportions after the larval period. Finally, hybridizations between brill (*Scophthalmus rhombus*) and turbot render monosex progenies, depending on the direction of the cross performed, which suggests different SD mechanisms in these congeneric species (Purdom and Thacker 1980).

In this study, we used the turbot genetic map (Bouza *et al.* 2007, 2008; Martínez *et al.* 2008) to look for sex-associated QTL in this species. The identification of a major QTL in a specific linkage group (LG) in the five families analyzed prompted us to refine the genetic map at this LG and to perform a comparative genomics approach against model fish species for a precise location and characterization of the putative SD region. Also, sex-associated QTL markers were screened in a large natural population to provide additional support to our findings and to obtain population parameters at sex-related markers that could aid in interpreting the evolution of this genomic region.

MATERIALS AND METHODS

Biological material

Families: The five families used to search for sex-associated QTL (Qfam) and to evaluate the association of specific markers with sex (Afam) were obtained from the genetic breeding program of the Stolt Sea Farm SA (SSF), a specialized turbot company located in northwestern Spain. Families were obtained following a three-generation scheme starting from unrelated grandparents coming from natural populations of the Atlantic Ocean. Two families were used for QTL identification using a large number of markers: Qfam1 (the DF reference family in Bouza *et al.* 2007) constituted 85 individuals (49 females and 36 males) and Qfam2 constituted 38 individuals (20 females and 18 males). Three additional families were used to confirm the QTL detected in Qfamilies by checking the association of the closest QTL markers to sex: Afam1 (39 individuals: 28 females and 11 males), Afam2 (30 individuals: 17 females and 13 males), and Afam3 (73 individuals: 36 females and 37 males).

Population: A total of 145 sexed breeders (50 females and 95 males) of the SSF broodstock were used to search for association of the closest QTL markers to sex at the population level. These breeders were collected in the Atlantic Ocean where very low or no significant genetic differentiation was previously reported in turbot (Bouza *et al.* 2002).

Sexing and DNA sampling: Qfamilies and Afamilies were selected depending on the suitability of the crosses and the availability of sexing information in progenies, respectively. Qfamilies were sexed at 8 months of age (~100 g and 18 cm) at Cluster de Acuicultura de Galicia facilities as soon as male and female gonads could be discriminated with confidence. Sex was recorded by examining gonad morphology after biopsy. Afamilies were constituted by 3-year-old fish, and sex could be determined by abdominal palpation at maturation time (an unambiguous procedure routinely practiced in turbot farms). A small piece of the caudal fin of each individual was cut and stored in absolute ethanol for DNA extraction.

Microsatellite genome scan: A total of 98 homogeneously distributed microsatellite markers previously described (Bouza *et al.* 2007, 2008) were analyzed in Qfam2. Average distances between these markers are 18.4 and 13.8 cM according to the total and framework turbot genetic map lengths, respectively (supporting information, Table S1). This

panel of markers is currently being used for identification of QTL related to productive traits in turbot. Qfam1 was one of the reference families for turbot mapping, and therefore 177 markers covering all LGs had been previously analyzed. Of these, 148 were anonymous (Bouza *et al.* 2007) and 29 were EST linked (Bouza *et al.* 2008). In this family, the 26 LGs reported in the turbot map were covered with at least 2 markers/LG and a mean of 6.5 markers/LG (Bouza *et al.* 2007). After QTL analysis, two additional microsatellite loci closely linked to the QTL detected were genotyped in Qfamilies to provide additional statistical support. Also, the 2–3 of the closest sex-associated QTL markers were analyzed in Afamilies and in the SSF broodstock to confirm QTL location and to look for association at the population level, respectively.

DNA was extracted from caudal fin clippings using standard phenol–chloroform protocols. Microsatellite PCR amplifications were carried out as previously reported (Pardo *et al.* 2006). Genotyping was conducted on an ABI 3730 DNA sequencer and analyzed using the Genemapper, version 3.7 software (Applied Biosystems, Foster City, CA). The complete cDNA sequence of the closest EST-linked microsatellite to the major sex-associated QTL was obtained following the ABI Prism BigDye Terminator v3.1 cycle sequencing kit protocol on an ABI 3730 DNA sequencer (Applied Biosystems).

Statistical procedures

QTL and sex-associated marker analysis: QTL analyses were performed using the software GridQTL 1.3.2 (Seaton *et al.* 2006) that considers the linkage phase between markers according to pedigree information. As each family arose from a single couple with a known genotype, the chosen module was the sib pair. The trait considered was sex (coded as a binary character: females—0; males—1), and no other fixed factor or covariate was included in the model. A single QTL was assumed at each linkage group. The default-solving method in the Grid QTL software (Haseman-Elston) was applied. Genomewide and LG-wide significant thresholds (for those linkage groups with a LOD score >2) were estimated by implementing a bootstrapping method at $P = 0.05$ and 0.01, respectively. The number of iterations was set to 1000. The Pearson χ^2 test was conducted to search for genotypic and allelic association between specific microsatellite markers and sex both in the families and in the SSF broodstock. Bonferroni correction was considered for multiple tests.

Genetic map refinement: The turbot genetic map (Bouza *et al.* 2007, 2008; Martínez *et al.* 2008) was reanalyzed at LG5, where the main sex-associated QTL was located (see results). Previous mapping data in the reference haploid (HF) and diploid (DF = Qfam1) families (Bouza *et al.* 2007) were revised, and missing data were supplied. Also, segregation data from Qfam2 and Afamilies, and from the other four diploid F_2 families currently used to look for QTL for tolerance to *Aeromonas salmonicida*, were used for map refinement at LG5. The order of adjacent triplets of markers was repeatedly tested using Joinmap 3.0 through an optimized algorithm to ensure marker order. The data files were screened for putative double recombinants, which were verified or corrected by reexamining genotypic data. A LOD threshold >3.0 and a recombination threshold <0.40 were used to obtain the framework map. The remaining markers were ordered by lowering the LOD threshold until they were included (in all cases the LOD was ≥2). Once the most likely order was obtained, genetic distances were estimated by applying the Kosambi mapping function (Kosambi 1944). The graphic maps were generated using MapChart 2.1 (Voorrips 2002). Genetic maps were constructed for each sex (averaging across the different families within sex), so recombination frequencies could be compared between male and female maps. A consensus LG5 map was constructed by using all segregation data with Joinmap 3.0 and by following the methodology previously reported (Bouza *et al.* 2007).

The position of the putative turbot sex-determining gene (SDg) was estimated by assuming that this was the only SD locus in the genome and that the trait showed full penetrance. For this, SDg genotypes of females and males were coded as heterozygotes and homozygotes, respectively, according to the ZW/ZZ model demonstrated in our study (see results). The mapping methodology outlined previously (Bouza *et al.* 2007) was applied.

The position of the centromere at LG5 was reanalyzed using previous data and new information obtained after genotyping 96 individuals of the reference diploid gynogenetic family (Martínez *et al.* 2008) with the closest informative centromere markers. Complete interference was used for estimating locus–centromere distances, and joint segregation analysis was applied to order the group of closely linked markers and the centromere (Thorgaard *et al.* 1983).

Comparative genomics of LG5: BLAST/Autofact searches of the SmaUSC-E30 sequence were performed against public databases for gene annotation. Additionally, unique sequences of the turbot genomic clones containing the microsatellite loci at LG5 were compared by NCBI-BLAST against model fish genomes downloaded from ftp://ftp.ensembl.org: *Tetraodon nigroviridis*, *Takifugu rubripes*, *Danio rerio*, *Oryzias latipes*, and *Gasterosteus aculeatus*. Hits were considered significant using a threshold of $E < 10^{-5}$ (Stemshorn *et al.* 2005).

Population analysis: The SSF broodstock was split by sex for analyzing population parameters at microsatellite loci. These were estimated in the whole population and in the male and female subsamples. In addition to the sex-associated QTL microsatellites analyzed in this work, previous data on 11 mapped microsatellites in the same population (Castro *et al.* 2004) were reanalyzed by sex to complete a panel of 20 microsatellites. These 11 microsatellites are essentially unlinked, and only Smax-02 and Sma3-129INRA map in the same LG at 34.8 cM. Expected heterozygosity (He) and the mean number of alleles per locus (A) were computed to estimate genetic diversity. Departure from Hardy–Weinberg proportions (HW) was checked by exact tests. The magnitude and sign of deviations at each locus were estimated by F_{IS} statistic. Genetic differentiation between male and female subsamples was estimated by using the relative coefficient of genetic differentiation (F_{ST}) and tested by using exact probability homogeneity tests. All these analyses were implemented using the default options of Genepop 3.1 (Raymond and Rousset 1995).

RESULTS

Sex-related QTL: All 177 microsatellites analyzed in Qfam1 were informative because this family had been used for mapping (Bouza *et al.* 2007, 2008). Among the 98 microsatellites analyzed in Qfam2, 79 were informative. Four QTL were detected in Qfam1 (qSD1, qSD2, qSD3, and qSD4) and only one in Qfam2 (qSD1) after a first analysis with 177 and 79 markers, respectively. The associations were maintained in a second-round analysis after including 2 additional closely linked microsatellites at all LGs where QTL had been detected (Table 1; Figure 1). A major highly significant QTL (qSD1) was detected close to the SmaUSC-E30 microsatellite at LG5 in both families. The association, although highly significant in both cases, was much higher in Qfam1

TABLE 1

Location, significance, and magnitude of the sex-associated QTL in turbot

						LOD threshold			
						Genomewide		LG-wide	
Family	QTL name	LG	Closest marker	LOD	*F* value	$P = 0.05$	$P = 0.01$	$P = 0.05$	$P = 0.01$
Qfam1	qSD1	5	SmaUSC-E30	1697.1	7815.6	25.9	52.5	13.6	30.6
	qSD2	6	Sma-USC110	5.1	23.7	25.9	52.5	15.4	38.6
	qSD3	8	Sma-USC59	3.9	17.4	25.9	52.5	11.7	26.9
	qSD4	21	Sma-USC231	4.3	19.7	25.9	52.5	7.4	21.8
Qfam2	qSD1	5	SmaUSC-E30	18.0	83	9.0	22.5	9.0	22.5

(LOD = 1697.1; $F = 7815.6$) than in Qfam2 (LOD = 18.0; $F = 83.0$). The SmaUSC-E30 marker correctly sexed 96.5% and 84.2% of individuals in Qfam1 and Qfam2, respectively. Additionally, three suggestive QTL were detected in Qfam1 at LG6 (qSD2), LG8 (qSD3), and LG21 (qSD4), which were close to Sma-USC110, Sma-USC59, and Sma-USC231 microsatellite loci, respectively. Their association to sex was significant only within the LG-significant threshold, but nonsignificant after correction for multiple tests. No additional QTL other than qSD1 were detected at Qfam2.

Association of sex-related QTL markers in Afamilies: Association of the four aforementioned QTL with sex was additionally checked in three families (Afam) using the two to three closest QTL-linked markers: SmaUSC-E30, Sma-USC270, and Sma-USC65 at qSD1; Sma-USC188 and Sma-USC110 at qSD2; Sma-USC194 and Sma-USC59 at qSD3; and Sma-USC117 and Sma-USC231 at qSD4. Association with qSD1 was detected in at least one of the tested markers in all families at both genotypic (g) $[P(\chi^2)_g = 0]$ and allelic (a) $[P(\chi^2)_a = 0]$ levels. This association was detected only with markers segregating in the mother (Table 2). Sma-USC270 in Qfam2 and Afam2 and Sma-USC65 in Afam1 and Afam3 did not show association with sex when segregation occurred only in the father. Markers showed significant

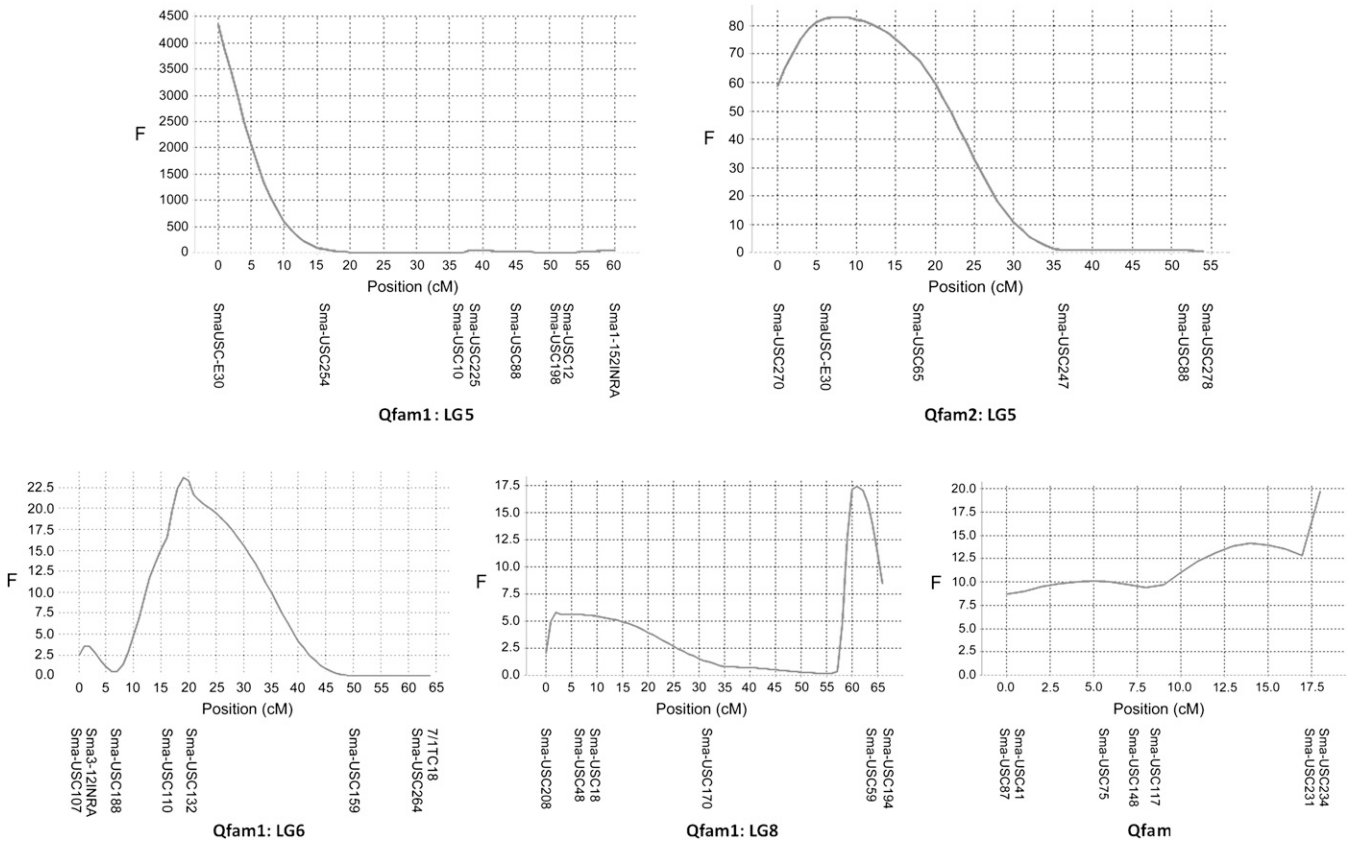

FIGURE 1.—Mapping of the sex-associated QTL in turbot. The estimated map positions of markers at each linkage group are indicated.

TABLE 2

Segregation of the three closest microsatellites to the major sex-associated QTL in turbot

Family	Microsatellite	Father	Mother	Offspring	
				Females	Males
Qfam2	SmaUSC-E30	179/179	179/181	179/179 (48) 179/181(1)	179/179 (2) 181/181(35)
	SmaUSC-270	311/311	311/311	ni	ni
	SmaUSC-65	140/140	142/142	ni	ni
Qfam2	SmaUSC-E30	181/183	183/185	181/- (3) 183/- (15)	181/- (17) 183/- (3)
	SmaUSC-270	307/311	311/311	307/311 (8) 311/311 (9)	307/311 (11) 311/311 (9)
	SmaUSC-65	128/128	128/138	128/128 (17) 128/138 (3)	128/128 (4) 128/138 (15)
Afam1	SmaUSC-E30	181/181	181/183	181/181 (0) 181/183 (28)	181/181 (11) 181/183 (0)
	SmaUSC-270	307/307	311/311	ni	ni
	SmaUSC-65	128/148	128/128	128/128 (14) 128/148 (14)	128/128 (6) 128/148 (5)
Afam2	SmaUSC-E30	181/181	181/185	181/181 (18) 181/185 (0)	181/181 (1) 181/185 (12)
	SmaUSC-270	301/307	311/311	301/311 (7) 307/311 (11)	301/311 (7) 307/311 (6)
	SmaUSC-65	128/128	142/148	128/142 (2) 128/148 (15)	128/142 (11) 128/148 (2)
Afam3	SmaUSC-E30	181/185	181/185	181/181 (13) 185/185 (0)	181/181 (1) 185/185 (17)
	SmaUSC-270	301/311	307/311	307/- (3) 311/- (35)	307/- (35) 311/- (6)
	SmaUSC-65	128/142	128/128	128/128 (18) 128/142 (19)	128/128 (15) 128/142 (24)

The number of males and females for each progeny are in parentheses. A hyphen represents an allele from the father. ni, non-informative cross

association even at long distances from qSD1, such as Sma-USC225 in Qfam1 [35.8 cM; $P(\chi^2)_g = 0$; $P(\chi^2)_a = 0$]. Association probabilities were much low at all other sex-associated QTL from Qfam1, where only the closest markers were significant. No sex association was detected with the closest markers to qSD2, qSD3, and qSD4 in the other four families analyzed (Qfam2, Afam1, Afam2, and Afam3).

Refinement of LG5 genetic map: The location of the putative SDg of turbot close to SmaUSC-E30 at LG5 moved us to refine the genetic map and to compare recombination frequencies between male and female genetic maps at this LG. The reanalysis of the mapping reference families (HF and DF; Bouza *et al.* 2007) and the increase of data from eight additional families (Qfam2, Afamilies, and four families used to identify QTL for tolerance to *A. salmonicida*) enabled us to obtain a more consistent order of markers at this LG (Figure 2). The number of framework markers increased from 8 to 11, but a much better definition was achieved especially at the extremes of this LG. The four closest markers to qSD1 (Sma-USC254, Sma-USC65, SmaUSC-E30, and Sma-USC270) are now framework markers. The length of this LG was reduced from 79.4 cM (Bouza *et al.* 2007) to 66.5 cM. Common pairs of segregating markers for comparison of recombination in male and female maps were available at four of the five closest markers to qSD1 (Sma-USC247, Sma-USC65, SmaUSC-E30, and Sma-USC270) in six families. No relevant recombination differences were detected between sexes. The only remarkable difference involved the SmaUSC-E30 and Sma-USC247 loci in Qfam2 (0.306 *vs.* 0.171 recombination frequency in female and male maps, respectively). Remarkably, the consensus map of Qfam2 suggested an inversion between the closest markers to SDg (Sma-USC270 and SmaUSC-E30).

A second goal within LG5 map refinement was to locate the positions of the putative SDg and the centromere. As shown in Table 2, the closest marker to SDg (SmaUSC-E30) appeared farther apart in Qfam2 ($r = 15.8$) than in the other four families (mean $r = 1.7$). The aforementioned inversion at Qfam2 could explain this observation. So to map SDg, we decided to exclude this family and to estimate the position of SDg using all

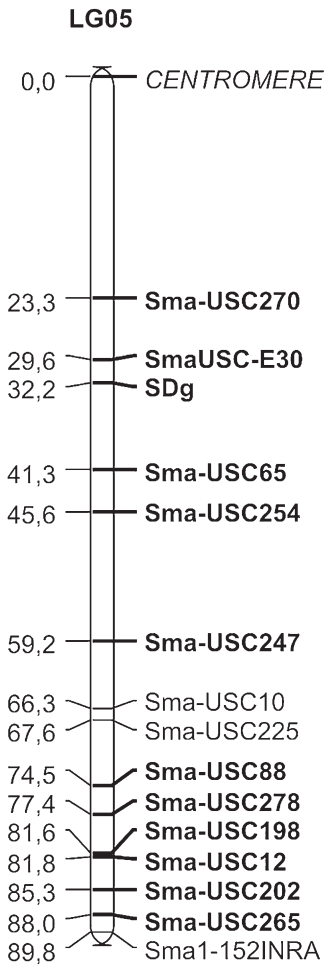

FIGURE 2.—Genetic map of turbot LG5. Framework markers (LOD >3) are presented in boldface type.

		Sma-USC65		
	Genotype	140/140	140/142	142/142
Sma-USC270	307/307	0	4	3
	307/311	1	25	0
	311/311	7	8	0

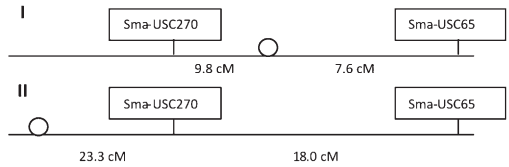

FIGURE 3.—Joint segregation analysis of the two closely linked turbot centromere microsatellites. I and II: alternative mapping positions of the LG5 centromere according to MARTÍNEZ *et al.* (2008) and present data, respectively. The centromere is represented by a circle.

informative markers of Qfam1 and the three Afamilies. For this, sex was considered a single-gene fully penetrant character, and SDg genotypes in females and males were coded as heterozygotes and homozygotes, respectively, according to the ZW/ZZ model demonstrated in this species. SDg was positioned at 32.2 cM from the centromere between SmaUSC-E30 and Sma-USC65 (Figure 2).

A more accurate location of centromere at LG5 was determined by analyzing a large sample (96 individuals) in the reference diploid gynogenetic family with the two closest informative markers to the centromere, Sma-USC270 and Sma-USC65 (MARTÍNEZ *et al.* 2008). An accurate centromere position could aid both in interpreting recombination frequencies in terms of physical distances in its vicinity and in explaining previous sex ratios observed in turbot gynogenetic and triploid progenies (CAL *et al.* 2006a,b). In Figure 3, the joint segregation analysis for both markers and the two alternative centromere locations—I (MARTÍNEZ *et al.* 2008) and II (present data) in Figure 3—is presented. Joint segregation evidenced the necessity of 25 double recombinants to explain the data under hypothesis I, while only 1 double recombinant would be necessary under hypothesis II.

Comparative genomics of LG5 microsatellites: The closest sex-associated microsatellite (SmaUSC-E30) was obtained from a 389-bp EST from a turbot EST database related to immune tissues (PARDO *et al.* 2008). The closeness of this EST to the putative SD region recommended its complete sequencing and subsequent bioinformatic analysis for gene annotation and for comparative genomics with related fish species (updated GenBank accession no. FE946656). No significant hits (*E*-value $<10^{-5}$) could be obtained either against public DNA, protein, and EST databases or against PROSITE (protein motifs) database.

BLASTn matches of 13 microsatellite sequences at LG5 against the *Tetraodon nigroviridis* (Tni), *Takifugu rubripes* (Tru), *Gasterosteus aculeatus* (Gac), *Oryzias latipes* (Ola), and *Danio rerio* (Dre) genomes revealed putative syntenic patterns with respect to these model fish species (Table 3). Matches appeared highly congruent because they involved the same microsatellites across different species following a decreasing homology from Gac to Dre. Nearly half of the turbot sequences compared showed significant homology against the Gac genome, four against the Tni and the Tru genomes (30%), two against the Ola genome (15%), and only one (8%) against the Dre genome. Significant matches ($E < 10^{-5}$) were due to small, highly conserved sequences between 22 and 252 bp (average 94 bp) in length and with 83 to 100% sequence similarities. Most matches were at the 20-cM distal region of turbot LG5 and represented putative syntenies of specific chromosomes (Tni LG1, Ola LG4, and Gac LG8) or chromosome regions (Tru scaffold-25) of the species compared. Among the query sequences of the LG5 proximal region, only the closest marker to SDg (Sma-USCE30) showed significant homology. This was achieved against the Gac genome (51 pb; 92% identity).

TABLE 3

Comparative analysis of turbot LG5 markers against model fish genomes

	Tru	Dre	Tni	Ola	Gac
SmaUSC-270	—	—	—	—	—
SmaUSC-E30	—	—	—	—	**LG8**
SmaUSC-65	—	—	—	—	—
SmaUSC-254	—	—	—	—	—
SmaUSC-247	—	—	—	—	—
SmaUSC-10	—	—	—	—	—
SmaUSC-225	**SC-332**	—	Unknown	—	**LG8**
SmaUSC-88	**SC-25**	—	**LG1**	—	**LG8**
SmaUSC-278	—	—		—	—
SmaUSC-198	—	—	—	—	—
SmaUSC-12	**SC-250**	LG8	**LG1**	**LG4**	**LG8**
SmaUSC-202	—	—		—	LG8
SmaUSC-265	**SC-25**	—	**LG1**	**LG4**	LG8

BLAST matches were against the *T. nigroviridis* (Tni), *T. rubripes* (Tru), *G. aculeatus* (Gac), *O. latipes* (Ola) and *D. rerio* (Dre) genomes using a significance threshold of $E < 10^{-5}$ (Stemshorn *et al.* 2005); most of them were retained at $E < 10^{-10}$ (in boldface type). "Unknown" refers to genome sequences that have not been mapped in Tni. SC, scaffold.

Sex association of markers in the natural population: The availability of a large sexed turbot population from the Atlantic Ocean allowed us to check the association of QTL markers with sex in a natural population and to estimate population parameters to analyze the evolution of the SD region. The existence of previous putatively neutral microsatellite data in the same sample (Castro *et al.* 2004) represented an appropriate material to be used as background for these analyses. Only 1 locus of 20 analyzed showed deviation from HW proportions after Bonferroni correction in the male (Sma3-129INRA) and female (Sma1-125INRA) subsamples and only 3 loci in the whole sample (Sma5-111INRA, Sma3-129INRA, and Sma-USC110) showed deviation (Table S2). Null alleles had been previously reported at the Sma3-129INRA locus after a detailed family analysis (Castro *et al.* 2004); this represents the most probable cause of positive deviations at this locus ($F_{IS} = 0.083$ and 0.053 in the male subsample and the whole population, respectively). Accordingly, only 3 of 60 tests (5%) deviated from the null hypothesis of HW proportions.

SmaUSC-E30, the closest to SDg, was the only locus among the 20 analyzed that showed significant sex association at genotypic [$P(\chi 2)_g = 0.033$] and allelic [$P(\chi 2)_a = 0.005$] levels, although not after Bonferroni correction (Table S2). This locus also was among the least diverse (He = 0.663; number of alleles = 5; mean He and A for all loci = 0.771 and 11.4, respectively; Figure 4, top; Table S2) and showed a significantly larger genetic differentiation between female and male subsamples ($F_{ST} = 0.0409$, $P = 0.008$) than the remaining loci (mean $F_{ST} = 0.0019$, $P = 0.427$) (Figure 4, bottom).

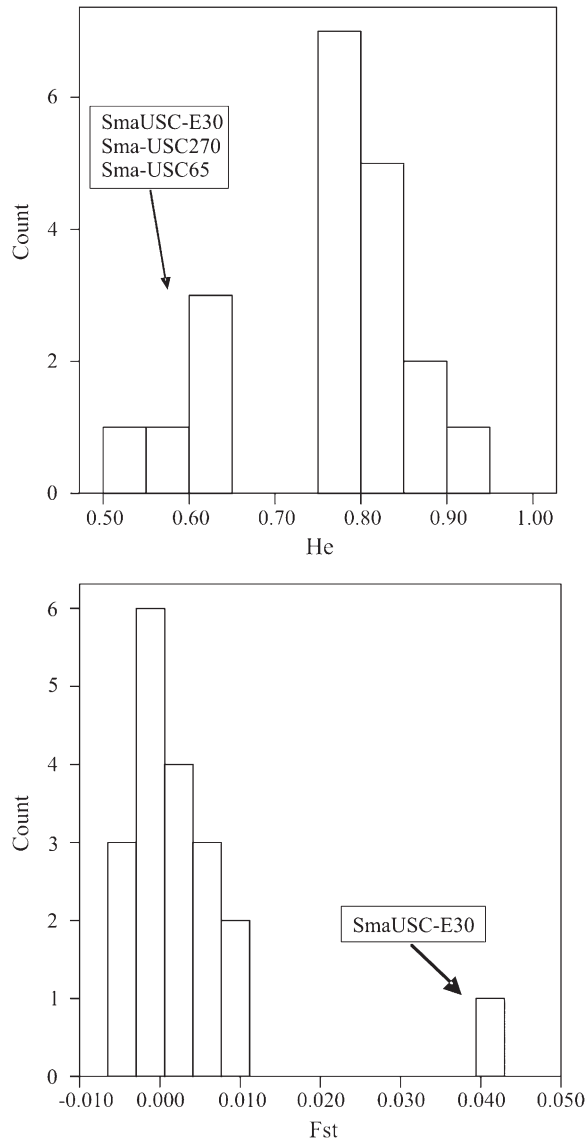

Figure 4.—Frequency histogram of microsatellite heterozygosity (top) and genetic differentiation (F_{ST}) between male and female subsamples of the turbot Atlantic Ocean population (bottom).

Another two close microsatellites to SDg (Sma-USC270 and Sma-USC65) were among the least variable loci (Figure 4, top).

DISCUSSION

The major SD region of turbot: In our study, a single major sex-associated QTL (qSD1) was detected in turbot at the proximal end of LG5. The association was highly significant even at very long distances (35.8 cM), and the closest marker to this QTL (SmaUSC-E30) correctly classified 98.4% offspring in four of five families analyzed. Another three minor sex-associated QTL were suggested at LG6, LG8, and LG21 in our analysis, but only in a single family and with low statistical support. SmaUSC-E30 also showed significant association with sex

in the panmictic natural turbot sample from the Atlantic Ocean. This was also the only locus where a significant differentiation between male and female subsamples was detected in this population. The F_{ST} value (4.1%) is close to that previously estimated among populations in the natural distribution of turbot, including the Atlantic Ocean and Mediterranean Sea areas (5–7%; Blanquer *et al.* 1992; Bouza *et al.* 1997). These observations support the close vicinity of the SmaUSC-E30 marker to the SDg in turbot, considering that the break in association between a pair of loci is directly related to the recombination frequency. Under a fully penetrant single-locus hypothesis, the turbot SDg was estimated to be 2.6 cM from the SmaUSC-E30 marker. This genetic distance would be even lower if other minor genetic and/or environmental factors were involved in turbot sex determination. This means that the SDg would be <1.4 Mb, considering the average relationship between physical and genetic distance in the turbot genome (0.53 Mb/cM; Bouza *et al.* 2007). In summary, our data strongly suggest that a major SDg is located at LG5 in turbot very close to the SmaUSC-E30 marker. Since this species has a simple ZW/ZZ sex-determination type (Haffray *et al.* 2009; this study), our data suggest that this gene is most likely the master SDg of turbot.

Insights on the turbot SD region from comparative genomics: Syntenies among species represent the bridge to complementing the initial QTL experiments with candidate gene approaches from homologous chromosomal locations identified in related model organisms (Erickson *et al.* 2004). In agreement with phylogenetic data, the comparative mapping of the 13 mostly anonymous turbot sequences at LG5 against model fish genomes showed higher similarities with other Acantopterygians such as *T. nigroviridis* (Tni), *T. rubripes* (Tru), *G. aculeatus* (Gac), and *O. latipes* (Ola) than with *D. rerio* (Ostariophysi; Miya *et al.* 2003; Li *et al.* 2008). The highest homology was observed with Gac, where homologous markers covered most of the LG5 length and included SmaUSC-E30, the SDg closest marker. Our data suggest the synteny of the turbot LG5 distal interval with Ola LG4, Gac LG8, and Tni LG1. The lack of homology of turbot LG5 markers with the sex chromosomes of medaka (LG1; Matsuda *et al.* 2002), stickleback (LG19; Peichel *et al.* 2004), or fugu (scaffolds anchored to LG19; Kikuchi *et al.* 2007) suggests that the sex chromosome of turbot evolved independently from that of these three model species. Nevertheless, turbot LG5 markers could be indirectly linked to the *Oreochromis* spp. LG23, where a sex-associated QTL was detected (Shirak *et al.* 2006) from a previous comparative homology demonstrated between *Oreochromis* spp. LG23 and stickleback LG8 (Sarropoulou *et al.* 2008). *Amh* and *Dmrta2* genes, involved in the gonadal differentiation pathway, map in the vicinity of the SD QTL at *Oreochromis* spp. LG23 (Shirak *et al.* 2006). These two genes also co-map to Gac LG8 (http://ensemble.org/index.html) and are physically located at ~10–13 Mb from the stickleback homologous sequence to the turbot Sma-USCE30, the closest turbot marker to SDg. These observations suggest a putative role of these genes in turbot sex determination and strongly recommend their mapping.

Comparison of sex determination with other Pleuronectiformes: Previous data in flatfish (Pleuronectiformes) suggest that a single genomic region is involved in sex determination, such as in turbot. This information was obtained mainly from sex ratios in progenies of meiogynogenetics and triploids, and both XX/XY and ZZ/ZW mechanisms have been reported (Purdom 1972; Tabata 1991; Howell *et al.* 1995; Tvedt *et al.* 2006; Chen *et al.* 2009). Environmental factors, such as temperature, do (Tabata 1995; Goto *et al.* 1999; Luckenbach *et al.* 2005) or do not affect (Hughes *et al.* 2008) gonad differentiation in flatfish, but this appears not to be a primary factor in sex determination in this group (Ospina-Alvarez and Piferrer 2008). Segregation patterns of the closest markers to the SD region in turbot support a ZZ/ZW mechanism in the five families analyzed. Our results are greatly in accordance with those reported by Haffray *et al.* (2009), who reported a ZZ/ZW mechanism in most turbot families obtained from androgen- and estrogen-treated parents crossed with normal females and males, respectively. Also, this mechanism would fit well with sex ratios of most triploid and meiogynogenetic families reported by Cal *et al.* (2006a,b). According to the fine mapping of the SD region obtained in our study, SDg would be at 32.2 cM from the centromere. This would render 82.2% female:17.8% male in meiogynogenetic (females: 64.4% ZW, 17.8% WW; males: 17.8% ZZ) and triploid (females: 64.4% ZZW, 17.8% ZWW; males: 17.8% ZZZ) progenies, assuming the dominance of the W chromosome and the normal viability of WW individuals. These proportions are very similar to those reported by Cal *et al.* (2006a,b). Cal *et al.* (2006b) invoked a primary XX/XY chromosome determinism in turbot on the basis of 100% all-female offspring obtained in a single meiogynogenetic family. However, as suggested by Haffray *et al.* (2009), this result also could be explained by a ZZ/ZW model that considers the presence of lethal genes associated with the SD region, as previously reported by Martínez *et al.* (2008).

Sex-associated heteromorphisms previously had not been detected either in mitotic or in the >11-fold longer meiotic chromosomes of turbot (Bouza *et al.* 1994; Cuñado *et al.* 2001). As in most fish species (Almeida-Toledo and Foresti 2001), this observation shows the primitive evolutionary condition of sex chromosomes in this species. In accordance with this observation, no consistent recombination differences were detected between males and females around the SD region in our study. However, these could be occurring at a finer scale as the significant genetic differentiation

(F_{ST} = 4.1%) at SmaUSC-E30 between males and females suggests. The brill (*S. rhombus*), a close related species according to genetic data (Blanquer *et al.* 1992; Pardo *et al.* 2001; Bouza *et al.* 2002), did not show any chromosome heteromorphism, and its mitotic karyotype was not distinguishable from that of turbot (Pardo *et al.* 2001). Remarkably, hybrid crosses between female brill × male turbot produce nearly all-male populations (Purdom and Thacker 1980). This could be explained by opposite sex determination mechanisms in both species (female XX × male ZZ). If so, a transition in the SD mechanism between these closely related species could have occurred recently. A similar situation has been suggested in tilapia species (Lee *et al.* 2004).

Other minor factors in turbot sex determination: The results discussed thus far explain most observations of sex determination reported to date in turbot. However, both in our study and in that by Haffray *et al.* (2009) some families did not conform exactly to the model proposed. In our work, SmaUSC-E30 did not predict the sex of individuals in Qfam2 as accurately as in the other families. The inversion suggested in the consensus map of Qfam2 could explain this discrepancy. Chromosome reorganizations in the SD regions in different species have been suggested as a way to suppress recombination to maintain sex-associated coadapted gene blocks (Peichel *et al.* 2004). Haffray *et al.* (2009) also reported some turbot families that did not conform to the ZZ/ZW model. An excess of males was observed in most of these families, with proportions close to 2 males to 1 female. Minor genetic or environmental factors could be necessary to explain these proportions. In this sense, a more detailed analysis of temperature during the most sensitive larval period could be undertaken in turbot for a better comprehension of the possible influence of temperature on sex ratios. Also, a QTL and marker association analysis in the atypical families reported by Haffray *et al.* (2009) could shed some light on this point.

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LITERATURE CITED

Alfaqih, M. A., J. P. Brunnelli, R. E. Drew and G. H. Thorgaard, 2009 Mapping of five candidate sex-determining loci in rainbow trout (*Oncorhynchus mykiss*). BMC Genet. **10:** 2.

Almeida-Toledo, L. F., and F. Foresti, 2001 Morphologically differentiated sex chromosomes in neotropical freshwater fish. Genetica **111:** 91–100.

Baroiller, J. F., and H. D'Cotta, 2001 Environment and sex determination in farmed fish. Comp. Biochem. Physiol. C. Toxicol. Pharmacol. **130:** 399–409.

Baron, D., R. Houlgatte, A. Fostier and Y. Guiguen, 2005 Large-scale temporal gene expression profiling during gonadal differentiation and early gametogenesis in rainbow trout. Biol. Reprod. **73:** 959–966.

Blanquer, A., J. P. Alayse, O. Berrada-Rkhami and S. Berrebi, 1992 Allozyme variation in turbot (*Psetta maxima*) and brill (*Scophthalmus rhombus*) (Osteichthyes, Pleuronectiformes, Scophthalmidae) throughout their range in Europe. J. Fish Biol. **41:** 725–736.

Bouza, C., L. Sánchez and P. Martínez, 1994 Karyotypic characterization of turbot (*Scophthalmus maximus*) with conventional, fluorochrome, and restriction endonuclease banding techniques. Mar. Biol. **120:** 609–613.

Bouza, C., L. Sánchez and P. Martínez, 1997 Gene diversity analysis in natural populations and cultured stocks of turbot (*Scophthalmus maximus* L.). Anim. Genet. **28:** 28–36.

Bouza, C., J. Castro, P. Presa, L. Sánchez and P. Martínez, 2002 Allozyme and microsatellite diversity in natural and domestic populations of turbot (*Scophthalmus maximus*) in comparison with other Pleuronectiformes. Can. J. Fish. Aquat. Sci. **59:** 1460–1473.

Bouza, C., M. Hermida, B. G. Pardo, C. Fernández, J. Castro *et al.*, 2007 A microsatellite genetic map in the turbot (*Scophthalmus maximus*). Genetics **177:** 2457–2467.

Bouza, C., M. Hermida, A. Millán, R. Vilas, M. Vera *et al.*, 2008 Characterization of EST-derived microsatellites for gene mapping and evolutionary genomics in turbot. Anim. Genet. **39:** 666–670.

Cal, R. M., S. Vidal, C. Gómez, B. Álvarez-Blázquez, P. Martínez *et al.*, 2006a Growth and gonadal development in diploid and triploid turbot (*Scophthalmus maximus*). Aquaculture **251:** 99–108.

Cal, R. M., S. Vidal, P. Martínez, B. Álvarez-Blázquez, C. Gómez *et al.*, 2006b Survival, growth, gonadal development, and sex ratios of gynogenetic diploid turbot. J. Fish Biol. **68:** 401–413.

Castro, J., C. Bouza, P. Presa, A. Pino-Querido, A. Riaza *et al.*, 2004 Potential sources of error in parentage assessment of turbot (*Scophthalmus maximus*) using microsatellite loci. Aquaculture **242:** 119–135.

Charlesworth, D., B. Charlesworth and G. Marais, 2005 Steps in the evolution of heteromorphic sex chromosomes. Heredity **95:** 118–128.

Chen, S. L., J. Li, S. P. Deng, Y. S. Tian, Q. Y. Wang *et al.*, 2007 Isolation of female-specific AFLP markers and molecular identification of genetic sex in half-smooth tongue sole (*Cynoglossus semilaevis*). Mar. Biotechnol. **9:** 273–280.

Chen, S. L., Y. S. Tian, J. F. Yang, C. W. Shao, X. S. Ji *et al.*, 2009 Artificial gynogenesis and sex determination in half-smooth tongue sole (*Cynoglossus semilaevis*). Mar. Biotechnol. **11:** 243–251.

Cnaani, A., N. Zilberman, S. Tinman, G. Hulata and M. Ron, 2004 Genome-scan analysis for quantitative trait loci in an F2 tilapia hybrid. Mol. Gen. Genomics **272:** 162–172.

Cuñado, N., J. Terrones, L. Sánchez, P. Martínez and J. L. Santos, 2001 Synaptonemal complex analysis in spermatocytes and oocytes of turbot, *Scophthalmus maximus* (Pisces, Scophthalmidae). Genome **44:** 1143–1147.

Devlin, R. H., and Y. Nagahama, 2002 Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences. Aquaculture **208:** 191–364.

Erickson, D. L., C. B. Fenster, H. K. Stenoien and D. Price, 2004 Quantitative trait locus analyses and the study of evolutionary process. Mol. Ecol. **13:** 2505–2522.

Felip, A., W. P. Young, P. A. Wheeler and G. H. Thorgaard, 2005 An AFLP-based approach for the identification of sex-linked markers in rainbow trout (*Oncorhynchus mykiss*). Aquaculture **247:** 35–43.

Goto, R., T. Kayaba, S. Adachi and K. Yamauchi, 1999 Effects of temperature on sex determination in marbled sole *Limanda yokohamae*. Fish. Sci. **66:** 400–402.

Haffray, P., E. Lebègue, S. Jeu, M. Guennoc, Y. Guiguen *et al.*, 2009 Genetic determination and temperature effects on turbot *Scophthalmus maximus* sex differentiation: an investigation using steroid sex-inverted males and females. Aquaculture **294:** 30–36.

Howell, B. R., S. M. Baynes and D. Thompson, 1995 Progress towards the identification of the sex-determining mechanism of the sole, *Solea solea* (L.), by the induction of diploid gynogenesis. Aquac. Res. **26:** 135–140.

Hughes, V., T. J. Benfey and D. J. Martin-Robichaud, 2008 Effect of rearing temperature on sex ratio in juvenile Atlantic halibut, *Hippoglossus hippoglossus*. Environ. Biol. Fish. **81:** 415–441.

Kallman, K. D., 1984 A new look at sex determination in Poeciliid fishes, pp. 95–171 in *Evolutionary Genetics of Fishes*, edited by B. J. Turner. Plenum, New York.

Kikuchi, K., W. Kai, A. Hosokawa, N. Mizuno, H. Suetake *et al.*, 2007 The sex-determining locus in the tiger pufferfish, *Takifugu rubripes*. Genetics **175:** 2039–2042.

Kondo, M., I. Nanda, U. Hornung, S. Asakawa, N. Shimizu *et al.*, 2003 Absence of the candidate male sex-determining gene *dmrt1b(Y)* of medaka from other fish species. Curr. Biol. **13:** 416–420.

Kosambi, D. D., 1944 The estimation of map distances from recombination values. Ann. Eugen. **12:** 172–175.

Lee, B. Y., G. Hulata and T. D. Kocher, 2004 Two unlinked loci controlling the sex of blue tilapia (*Oreochromis aureus*). Heredity **92:** 543–549.

Li, C., L. Guoqing and G. Ortí, 2008 Optimal data partitioning and a test case for ray-finned fishes (Actinopterygii) based on ten nuclear loci. Syst. Biol. **57:** 519–539.

Luckenbach, J. A., L. W. Early, A. H. Rowe, R. J. Borski, H. V. Daniels *et al.*, 2005 Aromatase cytochrome P450: cloning, intron variation, and ontogeny of gene expression in southern flounder (*Paralichthys lethostigma*). J. Exp. Zool. A Comp. Exp. Biol. **303:** 643–656.

Mank, J. E., D. E. L. Promislow and J. C. Avise, 2006 Evolution of alternative sex-determining mechanisms in teleost fishes. Biol. J. Linn. Soc. Lond. **87:** 83–93.

Marín, I., and B. S. Baker, 1998 The evolutionary dynamics of sex determination. Science **281:** 1990–1994.

Martínez, P., M. Hermida, B. G. Pardo, C. Fernández, J. Castro *et al.*, 2008 Centromere-linkage in the turbot (*Scophthalmus maximus*) through half-tetrad analysis in diploid meiogynogenetics. Aquaculture **280:** 81–88.

Matsuda, M., Y. Nagahama, A. Shinomiya, T. Sato, C. Matsuda *et al.*, 2002 DMY is a Y-specific DM-domain gene required for male development in the medaka fish. Nature **417:** 559–563.

Miya, M., H. Takeshima, H. Endo, N. B. Ishiguro, J. G. Inoue *et al.*, 2003 Major patterns of higher teleostean phylogenies: a new perspective based on 100 complete mitochondrial DNA sequences. Mol. Phylogenet. Evol. **26:** 121–138.

Ospina-Alvarez, N., and F. Piferrer, 2008 Temperature-dependent sex determination in fish revisited: prevalence, a single sex ratio response pattern, and possible effects of climate change. PloS ONE **3:** e2837.

Pardo, B. G., C. Bouza, J. Castro, P. Martínez and L. Sánchez, 2001 Localization of ribosomal genes in Pleuronectiformes using Ag- and CMA_3 banding and *in situ* hybridization. Heredity **86:** 531–536.

Pardo, B. G., M. Hermida, C. Fernández, C. Bouza, M. Pérez *et al.*, 2006 A set of highly polymorphic microsatellites useful for kinship and population analysis in turbot (*Scophthalmus maximus* L.). Aquac. Res. **37:** 1578–1582.

Pardo, B. G., C. Fernández, A. Millán, C. Bouza, A. Vázquez-López *et al.*, 2008 Expressed sequence tags (ESTs) from immune tissues of turbot (*Scophthalmus maximus*) challenged with pathogens. BMC Vet. Res. **4:** 37.

Peichel, C. L., J. A. Ross, C. K. Matson, M. Dickson, J. Grimwood *et al.*, 2004 The master sex-determination locus in three-spined sticklebacks on a nascent Y chromosome. Curr. Biol. **14:** 1416–1424.

Penman, D. J., and F. Piferrer, 2008 Fish gonadogenesis. Part I: genetic and environmental mechanisms of sex determination. Rev. Fish Sci. **16**(Suppl. 1): 14–32.

Piferrer, F., and Y. Guiguen, 2008 Fish gonadogenesis. Part II: molecular biology and genomics of sex differentiation. Rev. Fish. Sci. **16**(Supp1.): 35–55.

Purdom, C. E., 1972 Induced polyploidy in plaice (*Pleuronectes platessa*) and its hybrid with the flounder (*Platichthys flesus*). Heredity **29:** 11–24.

Purdom, C. E., and G. Thacker, 1980 Hybrid fish could have farm potential. Fish Farmer **3:** 34–35.

Raymond, M., and F. Rousset, 1995 GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. J. Hered. **86:** 248–249.

Ross, J. A., J. R. Urton, J. Boland, M. D. Shapiro and C. L. Peichel, 2009 Turnover of sex chromosomes in the stickleback fishes (Gasterosteidae). Plost Genet. **5:** e1000391.

Sarropoulou, E., D. Nousdili, A. Magoulas and G. Kotoulas, 2008 Linking the genomes of nonmodel teleosts through comparative genomics. Mar. Biotechnol. **10:** 227–233.

Seaton, G., J. Hernandez, J. A. Grunchec, I. White, J. Allen *et al.*, 2006 *GridQTL: A Grid Portal for QTL Mapping of Compute Intensive Datasets*. Proceedings of the 8th World Congress on Genetics Applied to Livestock Production, August 13–18, 2006. Belo Horizonte, Brazil.

Shirak, A., E. Seroussi, A. Cnaani, A. E. Howe, R. Domokhovsky *et al.*, 2006 *Amh* and *Dmrta2* genes map to tilapia (*Oreochromis* spp.) linkage group 23 within quantitative trait locus regions for sex determination. Genetics **174:** 1573–1581.

Stemshorn, K. C., A. W. Nolte and D. Tautz, 2005 A genetic map of *Cottus gobio* (Pisces, Teleostei) based on microsatellites can be linked to the physical map of *Tetraodon nigroviridis*. J. Evol. Biol. **18:** 1619–1624.

Tabata, K., 1991 Induction of gynogenetic diploid males and presumption of sex determination in the hirame *Paralichthys olivaceus*. Nippon Suisan Gakkaishi **57:** 845–850.

Tabata, K., 1995 Reduction of female proportion in lower growing fish separate from normal feminized seedlings of Hirame *Paralichthys olivaceus*. Fish. Sci. **61:** 199–201.

Tanaka, K., Y. Takehana, K. Naruse, S. Hamaguchi and M. Sakaizum, 2007 Evidence for different origins of sex chromosomes in closely related Oryzias fishes: substitution of the master sex-determining gene. Genetics **177:** 2075–2081.

Thorgaard, G. H., F. W. Allendorf and K. L. Knudsen, 1983 Gene-centromere mapping in rainbow trout: high interference over long map distances. Genetics **103:** 771–783.

Tripathi, N., M. Hoffmann, D. Weigel and C. Dreyer, 2009 Linkage analysis reveals the independent origin of Poeciliid sex chromosomes and a case of atypical sex inheritance in the guppy (*Poecilia reticulata*). Genetics **182:** 365–374.

Tvedt, H. B., T. J. Benfey, D. J. Martin-Robichaud, C. McGowan and R. Michael, 2006 Gynogenesis and sex determination in Atlantic halibut (*Hippoglossus hippoglossus*). Aquaculture **252:** 573–583.

Valenzuela, N., D. C. Adams and F. J. Janzen, 2003 Pattern does not equal process: Exactly when is sex environmentally determined? Am. Nat. **161:** 676–683.

Vandeputte, M., M. Dupont-Nivet, H. Chavanne and B. Chatain, 2007 A polygenic hypothesis for sex determination in the European sea bass *Dicentrarchus labrax*. Genetics **176:** 1049–1057.

Voorrips, R. E., 2002 MapChart: software for the graphical presentation of linkage maps and QTLs. J. Hered. **93:** 77–78.

Wilkins, A. S., 1995 Moving up the hierarchy: a hypothesis on the evolution of a genetic sex determination pathway. BioEssays **17:** 71–77.

Woram, R. A., K. Gharbi, T. Sakamoto, B. Hoyheim, L. E. Holm *et al.*, 2003 Comparative genome analysis of the primary sex-determining locus in salmonid fishes. Genome Res. **13:** 272–280.

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Identification of the Major Sex-Determining Region of Turbot (*Scophthalmus maximus*)

Paulino Martínez, Carmen Bouza, Miguel Hermida, Jesús Fernández, Miguel Angel Toro, Manuel Vera, Belén G. Pardo, Adrián Millán, Carlos Fernández, Román Vilas, Ana María Viñas, Laura Sánchez, Alicia Felip, Francesc Piferrer, Isabel Ferreiro and Santiago Cabaleiro

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TABLE S1

Microsatellite markers for QTL identification in turbot

Microsatellite	LG	Position
Sma-USC218	LG01	0.000
Sma-USC13	LG01	17.067
Sma-USC101	LG01	27.463
Sma-USC104	LG01	47.569
Sma-USC15	LG01	59.351
Sma-USC1	LG01	93.201
Sma-USC166	LG02	0.000
Sma-USC219	LG02	38.387
Sma-USC109	LG02	46.790
SmaUSC-E6	LG02	59.474
Sma-USC168	LG02	68.678
Sma-USC242	LG02	84.755
Sma-USC46	LG02	95.333
Sma-USC90	LG02	104.262
Sma-USC77	LG03	0.000
Sma-USC200	LG03	21.815
SmaUSC-E34	LG03	44.069
Sma-USC144	LG03	56.784
Sma-USC30	LG03	76.612
Sma-USC205	LG04	1.341
Sma-USC7	LG04	24.964
Sma-USC277	LG04	52.232
Sma-USC47	LG04	79.872
Sma-USC270	LG05	0.000
SmaUSC-E30	LG05	6.313
Sma-USC65	LG05	17.983
Sma-USC247	LG05	35.936
Sma-USC88	LG05	51.168
Sma-USC278	LG05	54.095
Sma-USC147	LG06	4.517
Sma-USC188	LG06	15.040
Sma-USC110	LG06	24.165

Sma-USC132	LG06	29.593
Sma-USC264	LG06	70.682
Sma4-14INRA	LG07	0.000
Sma-USC178	LG07	36.340
Sma-USC154	LG07	47.870
Sma-USC135	LG07	58.003
Sma-USC174	LG07	70.714
Sma-USC208	LG08	0.000
Sma-USC18	LG08	9.666
Sma-USC170	LG08	31.215
Sma-USC59	LG08	64.363
Sma-USC194	LG08	66.426
Sma-USC226	LG09	0.000
4/5CA22/6/2	LG09	5.712
SmaUSC-E41	LG09	22.956
SmaUSC-E23	LG09	37.891
SmaUSC-E36	LG09	53.238
Sma-USC150	LG09	66.007
Sma-USC79	LG10	1.132
Sma-USC162	LG10	18.426
SmaUSC-E32	LG10	32.398
Sma-USC217	LG10	55.241
Sma-USC22	LG11	7.735
Sma-USC62	LG11	22.380
Sma-USC158	LG11	31.939
Sma-USC201	LG11	52.282
3/9CA15	LG12	0.570
Sma-USC184	LG12	8.909
Sma-USC19	LG12	18.337
Sma-USC143	LG12	26.619
Sma-USC266	LG12	53.222
Sma-USC9	LG13	0.000
Sma-USC16	LG13	13.284
Sma-USC34	LG13	22.059

SmaUSC-E38	LG13	39.043
Sma-USC253	LG14	0.000
Sma-USC74	LG14	14.158
Sma-USC213	LG14	19.577
Sma-USC82	LG14	32.797
Sma-USC220	LG14	49.323
SmaUSC-E28	LG14	57.554
Sma-USC214	LG15	0.000
Sma-USC32	LG15	15.630
Sma-USC149	LG15	37.142
Sma-USC211	LG15	54.529
Sma-USC128	LG16	18.681
Sma-USC256	LG16	32.748
Sma-USC282	LG16	48.681
Sma-USC223	LG16	56.923
Sma3-8INRA	LG16	67.882
Sma-USC91	LG17	0.000
Sma-USC31	LG17	17.640
Sma-USC55	LG17	32.811
SmaUSC-E1	LG17	54.546
Sma-USC137	LG18	3.990
SmaUSC-E40	LG18	12.494
Sma-USC193	LG18	30.893
2/5TG14	LG19	6.438
Sma-USC23	LG19	17.458
3/20CA17	LG19	30.707
Sma-USC29	LG20	0.000
Sma-USC284	LG20	24.540
Sma-USC41	LG21	0.439
Sma-USC117	LG21	8.626
Sma-USC231	LG21	17.383
Sma-USC234	LG21	18.026

Sma-USC14	LG22	5.191
Sma-USC58	LG22	18.805
Sma-USC273	LG23	6.615
Sma-USC38	LG23	16.571
F8- I 11/8/17	LG24	2.749
Sma-USC100	LG25	0.039
Sma-USC175	LG26	0.000

Marker positions according to Bouza et al. (2007; 2008)

In bold characters markers genotyped for additional statistical QTL support

TABLE S2

Microsatellite population parameters by sex of the turbot Atlantic population

	LG	Position	Population	N	He	A	F_{IS}	F_{ST}	P(χ2)g	P(χ2)al
Smax-01	15	21,6	Males	95	0,802	13	0,095	0,0041	0,607	0,137
			Females	50	0,782	14	-0,049			
			All	145	0,797	15	0,048			
Smax-02	17	21,7	Males	95	0,841	23	-0,001	0,0049	0,333	0,699
			Females	50	0,778	16	-0,003			
			All	145	0,821	23	0			
Smax-03	3	11,3	Males	95	0,648	9	0,058	-0,0006	0,801	0,502
			Females	50	0,599	7	-0,035			
			All	145	0,631	9	0,028			
Smax-04b	19	38,9	Males	95	0,59	7	0,036	-0,0006	0,186	0,452
			Females	50	0,536	4	-0,081			
			All	145	0,571	7	-0,002			
Sma3-8INRA	16	49,4	Males	95	0,883	12	0,034	0,0029	0,494	0,143
			Females	50	0,854	12	0,063			
			All	145	0,874	14	0,045			
Sma5-111INRA	22	0	Males	95	0,893	16	-0,039	-0,0008	0,625	0,569
			Females	50	0,898	15	-0,047			
			All	145	0,894	18	**-0,042**			
Sma3-12INRA	6	9,6	Males	95	0,791	10	0,015	0,0087	0,226	0,353
			Females	50	0,808	10	-0,041			
			All	145	0,8	10	0			
Sma4-14INRA	7	84,2	Males	95	0,797	14	0,116	-0,0032	0,216	0,767
			Females	50	0,754	12	-0,034			
			All	145	0,781	14	0,065			
Sma1-125INRA	13	2,1	Males	95	0,769	10	-0,094	0,0042	0,095	0,537
			Females	50	0,806	8	**-0,17**			
			All	145	0,783	10	-0,119			
Sma3-129INRA	17	56,5	Males	95	0,918	20	**0,083**	-0,0017	0,623	0,657
			Females	50	0,918	20	-0,002			
			All	145	0,917	21	**0,053**			

Sma1-152INRA	5	43,9	Males	95	0,757	8	0,027	0,0012	0,614	0,265
			Females	50	0,736	8	-0,032			
			All	145	0,75	9	0,008			
Sma-USCE30	5	60,2	Males	91	0,663	5	0,038	0,0409	0,033	0,005
			Females	50	0,526	4	-0,066			
			All	141	0,626	5	0,026			
Sma-USC270	5	66,5	Males	88	0,5	3	0,115	-0,0063	0,493	0,174
			Females	49	0,52	3	0,097			
			All	137	0,506	3	0,106			
Sma-USC188	6	14,8	Males	95	0,829	11	-0,054	0,0101	0,401	0,118
			Females	50	0,758	10	0,131			
			All	145	0,809	11	0,011			
Sma-USC110	6	24,2	Males	95	0,842	14	0,037	0,0058	0,401	0,095
			Females	50	0,848	13	0,081			
			All	145	0,846	15	**0,055**			
Sma-USC194	8	66,4	Males	95	0,749	5	0,074	0,0034	0,643	0,237
			Females	50	0,748	5	0,093			
			All	145	0,751	5	0,082			
Sma-USC59	8	64,4	Males	94	0,778	12	-0,013	-0,0048	0,595	0,402
			Females	50	0,792	10	-0,062			
			All	144	0,781	14	-0,032			
Sma-USC117	21	8,6	Males	95	0,796	13	-0,071	-0,0005	0,307	0,741
			Females	50	0,822	13	-0,046			
			All	145	0,805	14	-0,063			
Sma-USC231	21	17,4	Males	95	0,808	12	-0,003	-0,0028	0,344	0,63
			Females	50	0,776	14	0,072			
			All	145	0,796	14	0,021			

Previously studied microsatellites are labeled in italics. N: number of individuals; He: expected heterozygosis; A: allele number; F_{IS}: within-population fixation index; F_{ST}: relative component of genetic differentiation. P(χ2)g and P(χ2) a: chi-square probability test for sex-association at genotypic and allelic levels, respectively. In bold characters significant F_{IS} at $P<0.05$ after Bonferroni correction.