Sex-associated DNA markers from turbot

LAURA CASAS1,2, LAURA SÁNCHEZ1 & LÁSZLÓ ORBÁN*2,3

1Departamento de Genética, Facultad de Veterinaria, Universidad de Santiago de Compostela, Campus de Lugo, Lugo, Spain, 2Reproductive Genomics Group, Temasek Life Sciences Laboratory, Singapore, and 3Department of Biological Sciences, National University of Singapore, Singapore

Abstract
The genomes of male and female turbot, Psetta maxima (Linnaeus, 1758), were screened for sex-specific sequences by comparative random amplified polymorphic DNA (RAPD) assays performed on pooled samples. As turbot females grow much faster than males, there is an increasing interest in differentiating between the sexes, and such markers would enable the sexing of individuals even at early stages of development. Four sex-associated DNA markers, with molecular sexing efficiencies ranging from 77 to 90%, were identified and further characterized. Two markers were identified from pooled female samples, one from pooled male samples (both sets collected from the wild), whereas a fourth marker was found in the female pool of farm-bred siblings. Parallel application of the three markers isolated from wild-caught individuals yielded a combined molecular sexing efficiency of 90% in males and 83.3% in females. While the three markers isolated from the wild should be useful to predict the sex in natural turbot populations, the fourth marker showed sex-association limited only to the family where it was isolated from. Nonetheless, it can be used for molecular sexing of brooders originating from the target family and potentially their offspring as well. The application of such molecular sex markers with limited scope is a potential strategy in aquaculture for those species for which no universal sex marker is available.

Key words: Fish sex, RAPD, SCAR, sex bias, sex chromosomes

Introduction
Turbot (Psetta maxima (Linnaeus, 1758); synonym: Scophthalmus maximus) is a marine flatfish species distributed all along the European coasts, from Norway to Morocco in the Atlantic area, and across the northern coasts of the Mediterranean up to the Black Sea (Blanquer et al. 1992). The severe decline of turbot fisheries during the last decades has been accompanied by a rapid increase in the aquaculture production of the species. As a result, turbot has become one of the main commercial species for marine aquaculture, not only in Europe (Villanueva & Souto 2000), but following its introduction, in northern China as well (Jilin 2003). Despite the obvious progress, important problems such as low larval viability, inbreeding depression, viral diseases and skewed sex ratios still persist in turbot culture (Bouza et al. 1997, 2002). Genome manipulations (Baynes et al. 2006; Cal et al. 2006a,b), hormonal sex reversal (Baynes et al. 2006; Haffray et al. 2009) and genetic technologies involving the use of DNA-based tools, including selective breeding programmes, DNA vaccines, and marker-assisted selection (see Rothschild & Ruvinsky 2007), are expected to offer solutions for some of these problems, enabling improvement in economically important traits for the aquaculture of the species (for reviews see Davis & Hetzel 2000; Dunham 2004).

During intensive farming, turbot females can exhibit over two-fold increase in their growth rate as compared to males (Husebye et al. 1994). Maturing turbot females were found to weigh 1.8 kg at 20 months of age, whereas the average weight of males at the same age was only around 1 kg (Piferrer et al. 2004). This is among the highest sex-related differential growth rates in cultured marine fish (Piferrer et al. 1995). The difference in the growth rate could be detected as early as 8 months after
hatching (Imsland et al. 1997) and it is maintained throughout the production cycle, even following sexual maturation (Piferrer et al. 2004). Under culture conditions, the males also mature earlier than females (Piferrer et al. 2003). In addition, turbot farming necessitates repeated grading in order to optimize feeding regimes and sexual maturation reduces somatic growth, increases mortality and susceptibility to diseases (Imsland et al. 1997; Piferrer et al. 2000). Altogether, these factors result in loss of production during the grow-out phase and, therefore, the production of all-female stocks is highly desirable. However, turbot is among those important commercial fish species where sexual dimorphism can only be observed late in the lifecycle, at the age when females outperform males in size (Stankus 2003), preventing the selection of one sex at early stages. Moreover, even at adulthood, it is difficult to estimate the sex of turbot visually and gonadal histology might be necessary for full confirmation.

Several different approaches have been tested to elucidate whether the sexual determination of turbot is based on a sex chromosomal system or not. Candidate genes and a Bkm repeat sequence (Singh et al. 1994) were tested in turbot, but none of them detected genetic differences between the two sexes (Husebye et al. 1994). Characterization of the karyotype using conventional banding techniques, fluorochromes and restriction endonuclease banding, has not revealed any sex-specific heteromorphisms (Bouza et al. 1994), while the synaptonemal complex (SC) analysed in spermatocytes and oocytes of turbot has also shown no evidence of a bivalent exhibiting atypical synaptic behaviour that could be associated with heteromorphic sex chromosomes in both sexes (Cuñado et al. 2002). Several hormonal sex reversal studies (Baynes et al. 2006; Haffray et al. 2009) and a gynogenetic experiment (Baynes et al. 2006) yielded data supporting the presence of ZW/ZZ sex chromosomes system. However, based on the analysis of triploids (Cal et al. 2006a) and gynogenotes (Cal et al. 2006b), others concluded that an XX/XY system is present.

The aim of the present study was to screen the male and female genome of turbot for sex-specific DNA markers that would allow for the molecular identification of the two sexes in turbot, even at early stages of the development. The use of such markers would allow for the potential improvement of turbot culture by opening the possibility of selecting females for the grow-out phase. We used comparative Random Amplified Polymorphic DNA (RAPD; Welsh & McClelland 1990; Williams et al. 1990) assays performed on pooled genomic DNA samples. This approach has been used before on the genome of several fish species, with variable success (Iturra et al. 1998; McGowan & Davidson 1998; Bardakci 2000; Kovacs et al. 2001; Li et al. 2002; Yue et al. 2003; Wuertz et al. 2006; Keyvanshokooh et al. 2007; Chen et al. 2009). This publication describes the isolation and characterization of four sex-associated RAPD markers from turbot.

Materials and methods

Sample collection

Samples of sexually mature turbot individuals were collected from different natural populations (A) and hatcheries (B) from the north-western region of Spain (Galicia). Sixty wild turbots (A) were caught from the Atlantic and Cantabric coasts of Galicia (Patos, Barra, Pontevedra and Xove, Lugo, respectively) while 20 farm-bred full-sibs (B) were provided by Stolt Sea Farm Company. The female brooder used to produce this family originated from a cross between two wild-caught individuals from the North Atlantic (Norway) (i.e. first generation), while the male brooder was from the second generation of the same lineage.

As the farmed individuals were used as brooders, they could not be sacrificed. Thus, their sex could only be estimated by obtaining gametes through abdominal massage. The fin clips of those producing gametes were collected, immersed immediately in 95% ethanol and stored at 4°C until use.

Wild-caught samples were anaesthetized using a 2% solution of ethyl-3-aminobenzoate methane sulfonate salt and subsequently sacrificed by cervical section. Animal care and sacrifice were performed according to the EU guidelines (European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS No. 123); Appendix A: Guidelines for Accommodation and Care of Animals (Article 5); 2006; http://conventions.coe.int/Treaty/EN/Treaties/PDF/123-Arev.pdf). Gonads were removed and fixed immediately in 3.6% neutral-buffered formalin. The fixed gonads were extracted, dehydrated, embedded in paraffin, sectioned at 3 μm and stained with Haematoxylin–Eosin for microscopical analysis.

DNA isolation

Fin clips were removed from ethanol, dried at room temperature for 15–20 s, and then placed into 2-ml Eppendorf tubes. Samples were digested in SSTNE extraction buffer (Blanquer 1990) with 0.1% SDS and 0.5 μg/ml proteinase K at 56°C overnight in a shaker. Genomic DNA was isolated using the standard phenol-chloroform method (Sambrook & Russell 2001).
The quality and concentration of genomic DNA was tested by spectrophotometry using either a UV/Vis Spectrophotometer UV Mini 1240 (Shimadzu Biotech, Kyoto, Japan) or a Nanodrop Spectrophotometer ND-1000 UV/Vis (Nanodrop Technologies, Wilmington, DE, USA) followed by agarose gel electrophoresis. Only DNA samples with clear electrophoresis profiles and ca. 20 ng/μl concentrations were selected for further analysis.

Preparation of DNA pools

Two different kinds of pools were prepared to be used in the search for sex-specific DNA sequences in turbot. The first pair of pools was generated by combining equal volumes of DNA samples, each with 20 ng/μl concentration from 11 wild-caught female and 11 wild-caught male individuals, respectively. The second set of pools was prepared using 10 hatchery-reared full-sibs of each sex to minimize the inter-individual variability not related to sex, and therefore, keeping individual variation of the genotypes at a reasonably low level, according to the bulk segregant analysis approach described by Michelmore and colleagues (1991).

Comparative RAPD assays performed on DNA pools

A total of 2030 10-mer RAPD primers were purchased from SIG Sigma Genosys (Woodlands, TX, USA), OP Operon Technologies (Alameda, CA, USA), the University of British Columbia (Vancouver, Canada) and Genemed Synthesis Inc. (San Francisco, CA, USA).

Two different RAPD protocols were used in our experiments. The first 520 primers (10-mers synthesized by Sigma Genosys) were tested on the wild-caught pools only following protocol #1 in Lugo, Spain. According to this protocol RAPD reactions were carried out in a total volume of 25 μl using the BIOTAQ™ DNA Polymerase package (Bioline, London, UK) containing 1 × PCR buffer, 2 μM RAPD primer, 100 μM dNTP mix, 2 mM MgCl₂, 20 ng total genomic DNA, and 0.5 U BIOTAQ polymerase.

Amplifications were performed in a PTC-100 thermal cycler (MJ Research, Watertown, MA, USA) by using the following programme: an initial denaturation at 94°C for 2 min followed by 35 cycles at 94°C for 15 s, 34°C for 45 s and 2 min at 72°C for extension. A final step was performed at 72°C for 5 min.

The other 1510 primers (Series AA to AZ and BA to BH from Operon, U001 to U559 from UBC and GS-1 to GS-250 from Genemed) were tested on all four pools in one of the Singaporean laboratories (TLL) using a different RAPD protocol (#2) described earlier by Bercsenyi and colleagues (1998).

PCRs were performed in a PTC-200 thermal cycler (MJ Research) by using the following conditions: 2 min at 85°C for preamplification denaturation; 20 cycles of 94°C for 20 s, 36°C for 20 s, 0.4°C/s to 72°C and 72°C for 5 min; 20 cycles of 94°C for 20 s, 36°C for 20 s, 1.5°C/s to 72°C and 72°C for 1 min 30 s; and then 72°C for 5 min for final elongation. This amplification programme introduced a slow heating/ramping at 1.5°C/s throughout, with the exception of the 36–72°C section, when it was slowed down to 0.4°C/s, because it has been reported that this procedure increased the number of amplified bands and enhanced reproducibility (Benter et al. 1995).

PCR products (20 μl) were separated on 2% agarose gels (Bio-Rad, Hercules, CA, USA) in 1 × TBE buffer containing either 0.5 μg/ml ethidium bromide or 10 nl/ml Gelstar (FMC BioProducts, Rockland, ME, USA). The gels were either placed onto a UV lamp and the band patterns were captured by a Polaroid camera or recorded by a DC120 Kodak Digital Camera (Eastman Kodak Company, Rochester, NY, USA). The resulting RAPD patterns were scored visually by two persons.

Confirming putative markers on individual samples

Putative markers have been further analysed by a multi-step process. First, every primer yielding a potentially sex-associated marker band was re-tested on the same pools and those which failed to reproduce the putative marker were excluded. Then the remaining primers were tested on the individual samples used earlier to generate the pooled DNA and those displaying percentages above 80% of presence/absence of the polymorphism at least in one of the sexes, were selected for further analysis. This is the proportion that would yield statistically significant results when a contingency exact test with the sample size used in our experiments was applied (chi square test 95% confidence interval, x = 0.05).

Isolating, cloning and sequencing of the markers

The selected sex-associated and control bands were excised from the gel and their DNA content was isolated using the GFX™ PCR DNA and Gel Band Purification Kit (Amersham Biosciences, Piscataway, NJ, USA). Each fragment was then ligated into the pGEM-T Easy cloning vector (Promega, Madison, WI, USA).

For every marker (and corresponding control band) four clones containing an insert of the
Correct size were grown in liquid media, purified by using the QIAprep Spin Miniprep Kit (Qiagen, Chatsworth, CA, USA) and sequenced on both strands with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s recommendations in an ABI Prism 3100 sequencer (Perkin Elmer, Foster City, CA, USA). Sequencing reactions were carried out in a total volume of 20 μl, containing 2 μl 5 x BigDye sequencing buffer, 4 μl 2.5 x Terminator Ready Reaction Mix, 3.2 pmol universal primer T7 or SP6, and 1 μl of purified DNA. Consensus sequences were produced using Clustal X software (ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX, version 1.83), and BLAST searched (Altschul et al. 1990) against GenBank (http://www.ncbi.nlm.nih.gov/blast).

Conversion into SCAR markers

Specific PCR primer pairs (18–22-mer) were designed for each cloned RAPD product, using the Primer3 software (Rozen & Skaletsky 2000; available at http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi), to convert them into sequence characterized amplified region (SCAR) markers (Paran & Michelmore 1993). Primers were located as close to the ends of the markers as possible — including the original RAPD primer — depending on the sequence.

PCR reactions were set up and optimised according to Henegariu and colleagues (1997). Primer pairs and conditions yielding clear bands were selected. (Primer sequences and amplification conditions are available from the authors upon request.)

Searching for sex-specific differences within the bands amplified by specific primers

Each SCAR product was amplified from three individuals, which scored positive for the corresponding original RAPD marker and from three ‘negative’ individuals from the opposite sex which did not, under conditions optimized previously. PCR products were purified by using the Spin-Clean™ PCR Purification Kit (Mbiotech Inc., Seoul, Korea) and directly sequenced with conditions and protocol described earlier, but replacing the universal primers with the specific ones in the sequencing reaction. Sequence editing, fragment assemblies and generation of consensus sequences were performed by using the Clustal X software. The same program was used to search for sex-associated differences among the ‘positive’ and ‘negative’ individuals.

Results

Comparative RAPD assays on pooled turbot DNA samples

We have performed a comparative RAPD analysis on two pairs of pooled DNA samples from turbot males and females. The first two pools contained DNA from wild-caught male and female individuals, whereas the other two were from siblings obtained from a farm-bred family. The comparative assay of the pooled samples yielded a total of 158 putative sex-discriminating markers (34 from the first set and 124 from the second set).

Testing of putative markers on pooled and individual samples confirms four sex-associated DNA markers from turbot

Primers yielding a potentially sex-associated marker band were re-tested on the same pools by running two additional pairs of reactions. In 138 out of the 158 cases at least one of the patterns was not consistent with the previous one due to extra band(s), missing band(s) or entirely different pattern. These were excluded from further analysis. When the remaining 20 primers were tested on the individual samples, four sex-associated RAPD markers were identified (Figure 1). The first three (Sma1Fe, Sma2Fe and Sma3M) were amplified from pools generated from the wild population.

Figure 1. The four sex-associated RAPD markers isolated from pooled turbot samples. Samples from the left: Lane (1) 100-bp ladder (Amersham Pharmacia); Lanes (2–7) RAPD patterns produced from the wild-caught pools by primers SiG01, OP-E01 and OP-BE12; 8: 40X174 DNA/Hae III Marker (Promega); Lanes 9-10) RAPD patterns amplified from the farm-bred pools by primer OP-AS13; Lane (11) 100-bp ladder (Amersham Pharmacia). In all four pairs of pools the female one is located on the left. Arrows indicate the putative sex-specific markers.
whereas the fourth (Sma4Fe) was from the farmbred family.

**Marker Sma1Fe.** This 300-bp marker was detected in the pattern amplified by primer SiG01 from the female pool of wild-caught individuals, but it was absent from the pattern of the male pool (Figure 1). When re-tested on the wild-caught individual samples of the pool, the marker was amplified from nine out of 11 females and only four out of 11 males (81.8% and 63.6% sexing efficiency, respectively). The extended test on 19 additional individuals of each sex from the wild-caught population (a total of 30 individuals per sex) confirmed the sex association in 83.3% of the females and 70% of the males, thus yielding a 76.7% combined sexing efficiency for both sexes (Figure 2, Supplementary Table S1, available on the Supplementary Content tab of the article’s online page at http://dx.doi.org/10.1080/17451000.2010.515226).

**Marker Sma2Fe.** The second marker of ca. 800 bp was amplified by primer OP-E01 (Figure 1). Similar to Sma1Fe, this marker was also present in the pattern amplified from the wild-caught female pool, but absent from the male pool. In individual samples present in the pool, the marker was amplified from nine out of 11 females and none out of 11 males, yielding an 81.8% and 100% sexing efficiency for females and males, respectively (90.9% combined efficiency for both sexes). When tested on 19 additional individuals per sex from the wild population, 83.3% of the females and 90% of the males were correctly sexed in total, yielding a 86.7% combined sexing efficiency for both sexes (Supplementary Figure S1, Supplementary Table S1, available on the Supplementary Content tab of the article’s online page at http://dx.doi.org/10.1080/17451000.2010.515226).

**Marker Sma3M.** The third marker of 1100 bp was isolated from the wild-caught pools through amplification with primer OP-BE12. Contrary to the markers described above, this marker was present in the pattern amplified from the male pool but absent from the female one (Figure 1). When tested on individual samples present in the pool, the marker was amplified from nine out of 11 males and two out of 11 females only (81.8% sexing efficiency for both sexes). Further tests on 19 additional individuals per sex from the wild population confirmed the sex association, with 83.3% of the males and 80% of the females showing the expected profile, yielding an 81.7% combined sexing efficiency for both sexes (Supplementary Figure S2, Supplementary Table S1, available online).

**Figure 2.** The Sma1Fe RAPD marker associated with femaleness identified the sex of wild-caught individuals with 76.7% efficiency. RAPD patterns were produced by primer SiG01. Panel A: Patterns from wild-caught female samples present in the pool (lanes 2-12) and additional wild-caught females not present in the pool (lanes 13-16 and 19-33). Panel B: Patterns from wild-caught males present in the pool (lanes 2-12) and additional wild-caught males not present in the pool (lanes 13-16 and 19-33). Molecular weight standards: 100-bp ladder (Amersham Pharmacia) in lanes 1 and 18 as well as FX174 DNA/Hae III Marker (Promega) in lanes 17 and 34. Arrows indicate the confirmed sex-associated marker detected in individual samples.
Marker Sma4Fe. The last marker of ca. 600 bp was produced by primer OP-AS13 (Figure 1). This band was present in the RAPD pattern amplified from the female pool of the farm-bred family but absent from the male one. When tested in the full-sib individuals the marker was amplified from 8 out of 11 females and none out of 11 males (Supplementary Figure S3), yielding a 73% sexing efficiency for females and 100% for males (86.5% combined for both sexes). It was not possible to test this marker on additional full-sibs, since by the time the marker was isolated the rest of the family had been sold by the farm.

When the first three markers were applied in combination for the analysis of the 60 wild-caught specimens, the following results were obtained: for 35 individuals (19/30 females and 16/30 males) data obtained with all three markers have shown full agreement with each other and with the phenotypic sex. Of the remaining 11 females the phenotype of 6 showed agreement with 2 markers and 5 with only 1, whereas from the remaining 14 males the phenotype of 11 showed agreement with 2 markers and 3 with only 1 (Supplementary Table S1). There was no single individual for which the phenotypic sex disagreed with the molecular sex obtained with all three markers.

Unfortunately, none of the markers isolated from the wild population could be amplified from the farm-bred individuals. Similarly, the marker associated with phenotypic female characters in the farm-bred stock did not show sex association in the wild population.

Cloning of the four sex-associated RAPD markers from the turbot genome

The sex-associated RAPD markers were re-amplified from one to two individuals each, isolated from the gel and cloned into a plasmid vector. Inserts were sequenced from four clones per marker, yielding the following results: Sma1Fe – 296 bp (GenBank ID: EF612192); Sma2Fe – 792 bp (EF612194); Sma3M – 1109 bp (EF612196) and Sma4Fe – 598 bp (EF612198).

When BLAST-ed against GenBank, the first three sequences did not show any significant match either at DNA or protein level (e-values <0.05). However, the translated protein sequence corresponding to the first 380 bp of Sma4Fe marker turned out to be highly similar (e-values: from 8e-54 to 6e-36) to a number of vertebrate alpha-1b adrenergic receptor sequences, among them those of rainbow trout, Onchorhynchus mykiss (Walbaum, 1792); EF667964.1), zebrafish, Danio rerio (Hamilton-Buchanan 1822); XP_686768) and green spotted pufferfish, Tetraodon nigroviridis (Marion de Procé, 1822); CAF97256) (the latter two being indicated as hypothetical or unnamed proteins, respectively).

Converting the RAPD markers into SCAR markers and recovering the sex-associated polymorphism in one product

We converted all four sex-associated RAPD markers to specific SCAR markers (Paran & Michelmore 1993) in an attempt to improve their user-friendliness. However, when the SCAR markers were re-tested on individual samples their initial sex-associated polymorphism was lost in all four cases, and the marker-specific bands were amplified indiscriminately in all individuals.

In an attempt to recover the original sex-associated polymorphism, we sequenced the SCAR markers and searched the resulting sequences for sex-associated polymorphisms. Data showed the presence of at least one SNP or indel in every marker (data not shown). However, only in one of the cases, in the sequence of marker Sma4Fe, did we find a sex-associated difference in the form of a single-base deletion in all three female individuals tested (data not shown; available from the authors upon request).

Discussion

On the potential use of the four sex-associated RAPD markers

Sex-specific DNA markers with molecular sexing ability of different efficiency in various strains have been reported previously from several species (e.g. Yue et al. 2003; Chen et al. 2009).

In this study, we have been searching for general sex-specific DNA markers that would allow for molecular sexing of all turbot individuals by using a PCR-based ‘blind’ assay, RAPD. We have identified four sex-associated markers showing an agreement with the sexual phenotypes in 76.7–86.7% of the individuals tested. The first three markers isolated from wild-caught individuals are expected to allow for the sexing of individuals from natural origin as a high level of genetic similarity has been revealed among the natural populations in turbot and historical bottlenecks have been hypothesized (Bouza et al. 1997, 2002). The fourth marker is able to predict the sex of individuals only in the family where it was isolated from. Such a marker would likely allow for molecular sexing of the offspring.
from the same set of brooders that were used for the isolation of markers. Repeated crossing of these brooders would likely yield offspring groups for which the sex ratios could be estimated and individuals could be sorted according to their future phenotypic sex even prior to their maturation.

Despite our expectations, more markers have been found in the wild-caught samples, than in the farm-bred stock. The potential reason for this could be a homogenization effect of inbreeding on the poorly differentiated, extensively recombining sex chromosomes in the latter. On the other hand, the sex association of several markers in the wild population suggests the existence of gametic disequilibrium, and therefore that markers might be closely linked with the sex-determining gene(s). Such disequilibrium should be maintained in the family, provided that existence of the appropriate variation allows for the analysis.

**Turbot hasZW/ZZ sex chromosomes that appear to be poorly differentiated**

During the past 10 years several groups have performed experiments to find out more about turbot sex. Genome manipulation (for reviews see Felip et al. 2001; Komen & Thorgaard 2007) and hormonal sex reversal experiments (for review see Pandian & Kirankumar 2003) were used to obtain indications about the presence of strong sex chromosomal systems. In four groups of gynogenetic turbot individuals the proportion of females was found to be 69, 75, 90 and 100% (Baynes et al. 2006; Cal et al. 2006b). Based on their data, Cal and colleagues (2006a,b) argued that the fact that only females were obtained in one of their gynogenetic families provided proof of female homogamety (XX/XY) in turbot. On the other hand, Baynes et al. (2006) rejected the simple XX/XY system based primarily on the presence of 35% males among the progeny of masculinized gynogens crossed with normal females.

Haffray and colleagues (2009) have generated both sex-reversed neo-males and neo-females, respectively, by hormonal treatments, crossed them with wild-type partners and analysed the sex ratio of their offsprings. The sex ratios of most families fit a ZZ/ZW genetic model; on the other hand, one-third of them could not be explained by an XX/XY model.

Shortly before the submission of this publication, a paper describing the QTL-based mapping of a marker associated with the major sex determining region of turbot onto this map was published online (Martinez et al. 2009). It showed that turbot has a ZW/ZZ chromosomal system and described a sex-associated microsatellite marker. Our data seem to be in agreement with this finding, as three of the four markers we have identified were found in the female pool. (The fourth one, associated with maleness, might be a modifier gene with pro-male effect segregating in some of the families.)

Our data also allow us to speculate on the level of differentiation of turbot sex chromosomes. Assuming that RAPD primers on average produce 10 bands per reaction (Liu & Cordes 2004) that are randomly distributed along the genome and are also unrelated, the estimated number of loci tested in our experiment is 20,300. Since the turbot genome is quite small (C-value: 0.65–0.86 pg; ~ 636–841 Mb estimated genome size; Cuñado et al. 2002; Hardie & Hebert 2003, 2004), that would yield an estimated average distance of 31.3–41.4 kb between markers. This seems to indicate that the differential (i.e. non-recombining) region between the turbot sex chromosomes is rather short, in concordance with the absence of differences between male and female karyotypes and between the synaptonemal complexes in spermatocytes and oocytes in the species (Bouza et al. 1994; Cuñado et al. 2002).

**The potential usefulness of a sex-associated marker located in the alpha-1b adrenergic receptor locus**

Bioinformatic analysis of the four marker sequences has identified high level of sequence similarities between the first 380 bp of *Sma4Fe* marker and the alpha-1b adrenergic receptor gene of several teleost species, indicating that we have identified one or more exons of the corresponding locus from the turbot. As the binding of RAPD primers to the genomic DNA is not targeted to exons, they typically bind to non-coding intergenic and intronic sequences that make up the vast majority of eukaryotic genomes. Therefore, reports on RAPD products amplified from open reading frames (ORFs) are rather scarce (see Garcia et al. (1996) and Nakao et al. (2002) for exceptions). Moreover, most of these ORFs are without known function. Our result adds one more case to this short list, and might help with mapping of the locus and the isolation and characterization of its mRNA product in turbot.

Recent data obtained from knockout mice indicate that alpha 1b adrenergic receptor signalling is essential for male fertility in mammals, as in the absence of the functional product both spermatogenesis and the steroidogenic capacity of the Leydig cells are compromised (Mhaouty-Kodja et al. 2007). Moreover, data from the study of Itoh & Ishizuka (2005) also indicate that alpha1 adrenergic receptors seem to play an important role in ovarian function as well.
Having a sex-associated marker in a gene with important function in both gonad types might open up interesting possibilities not only in molecular sexing, but in marker-assisted selection work on turbot as well.

**How can we improve the efficiency of sex marker identification and their conversion to SCAR markers?**

The RAPD assay is useful for the detection of differences between two complex, unknown DNA samples, since it is easy to set up and learn, inexpensive and fast. However, RAPD markers are often sensitive to modification in PCR reaction conditions, resulting in variable reproducibility (MacPherson et al. 1993; Meunier & Grimont 1993; Jones et al. 1997; Liu & Cordes 2004). For this reason, researchers have used several alternative methods for detecting differences between complex DNA samples, including Amplified Fragment Length Polymorphism (AFLP; Vos et al. 1995), Represenntational Difference Analysis (RDA; Lisitsyn et al. 1993; Lisitsyn 1995) and Suppressive Subtractive Hybridization (SSH; Bogush et al. 1999; Nesbo et al. 2002). Nonetheless, comparative RAPD assays have yielded more sex-specific markers than any of the previous methods: they have been used successfully in rainbow trout (Iturra et al. 1998); Nile tilapia (Linnaeus, 1758) (Bardakci 2000); African catfish, Clarias gariepinus (Burchell, 1822) (Kovacs et al. 2001); Asian arowana, Scleropages formosus (Müller & Schlegel, 1844) (Yue et al. 2003) and common carp Cyprinus carpio (Linnaeus, 1758) (Chen et al. 2009) among other species. In those species, where a relatively large set of RAPD assays failed to yield sex markers (e.g. Li et al. 2002; Wuertz et al. 2006; Keyvanshokooh et al. 2007), the potential lack of sex chromosomes is considered.

Recently, a new genotyping method called fluoMEP has been described and recommended for such screens (Chang et al. 2007). The new procedure is based on RAPD, but the inclusion of a fluorescently labelled ‘common primer’ makes it compatible with high-throughput detection, thereby reducing the number of reactions needed to search for sex specificity in a given number of PCR products. Moreover, the ‘common primer’ can be designed to bind to frequently occurring motifs in the genome, opening the possibility for targeting the products onto particular regions of the genome (Chang et al. 2007). Preliminary data from the Singaporean lab indicate that fluoMEP is indeed an efficient tool for the isolation of sex markers (Liew, Lim & Orban, unpublished data). We propose that fluoMEP should be utilized for the searches aiming to identify sex markers from turbot and other species with similar characteristics.

Our attempts to transform the isolated RAPD markers into SCAR markers, less sensitive to PCR reaction conditions, were unsuccessful as the sex specificity was lost in the process. Similar experiences were reported earlier by several authors (e.g. Deng et al. 1997; Zhang & Stommel 2001). RAPD polymorphisms can occur due to either base substitutions or small deletions/insertions at the primer binding sites or to indels in the region between the sites (Liu & Cordes 2004). In the first case, the elongation of the original RAPD primers would result in longer primers with the SNP close to the 5’ end of the former preventing the selective binding. Therefore, successful conversion of sex-specific RAPD markers that are due to SNPs or small indels in the primer binding site to SCAR markers would require the extension of these markers into their flanking region to the 5’ direction by genome walking.

A medium-density genetic linkage map was published for the turbot earlier without indication on the sex chromosomes (Bouza et al. 2007). The W-linked microsatellite marker isolated by Martinez and colleagues (2009) was able to assign correctly the sex in the 96.5% and 84.2% of the individuals from two farm-bred families, respectively. When tested on natural populations, however, this marker showed significant sex-association at genotypic and allelic levels, although not after Bonferroni correction’ (Martinez et al. 2009). Therefore, we feel that the sex-associated RAPD markers described in our paper might provide additional useful resources for molecular sexing of wild-caught turbots.

**Acknowledgements**

The authors would like to thank Stolt Sea Farm S.A. (Lira, Spain) for providing farmed turbot samples. This project was funded by the Ministry of Education and Science of Spain (Grant No.: AGL2003-05539), by the Xunta de Galicia Local Government (Grant No: PGIDIT04PXIC26104PN) and by an internal research grant from Temasek Life Sciences Laboratory (Singapore). LC’s internship at TLL was financially supported by the General Direction of Research, Development and Innovation of the Xunta de Galicia Local Government through a Human Resources Program Grant.

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Editorial responsibility: Franz Uiblein