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A Cryptic RRY(i) Microsatellite From Atlantic Salmon (*Salmo salar*): Characterization and Chromosomal Location

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In this article we describe the isolation and characterization of a cryptic RRY(i) microsatellite from an Atlantic salmon genomic cosmid library. The chromosomal location of the microsatellite-containing cosmid was performed by fluorescent in situ hybridization (FISH) showing a single-locus signal located on an interstitial position of an acrocentric pair. The suitability of this type of microsatellite marker for population genetic analysis and for the development of a genetic map in this species is discussed. In addition, the usefulness of cosmid libraries for physical mapping of microsatellite markers and therefore for the integration of physical and genetic maps is pointed out.

Most eukaryotic genomes contain a considerable number of repetitive noncoding sequences that exist as both dispersed copies and tandem arrays. Microsatellites (tandemly repeated motifs of 1–5 bp) belong to this second category.

Microsatellite loci can be defined by their specific flanking sequences showing a high degree of length polymorphism (Weber 1990), which can be analyzed by the polymerase chain reaction (PCR) followed by sizing on polyacrylamide gels (Weber and May 1989). This polymorphism, joined with their even and apparently random distribution in the genome, makes microsatellite loci very useful as markers for genetic mapping and identity control and they have been used for the development of high-resolution genetic maps of species such as human and mouse (Chapman and Nadeau 1992; Weissenbach et al. 1992). Low-resolution genetic marker maps, also based on microsatellite markers, are being developed in a wide variety of commercially important species, such as pig, chicken, cattle, rainbow trout, tilapia, and flat oyster (Buchanan et al. 1993; Kocher et al. 1998; Moran 1993; Naciri et al. 1995; Rohrer et al. 1994; Young et al. 1998).

Genetic linkage maps are complemented with physical mapping, which enables the assignment of linkage groups to specific chromosomes (Ellegren et al. 1994; Toldo et al. 1993). The development of fluorescent in situ hybridization (FISH) using microsatellite-containing cosmids as probes has been an important advance and has been used in different map projects (Dickens et al. 1999; Fischer et al. 1996; Toldo et al. 1993). This method is of particular importance in species, like fishes, whose karyotypes are not standardized, since FISH can simultaneously allow chromosome identification and genetic data integration.

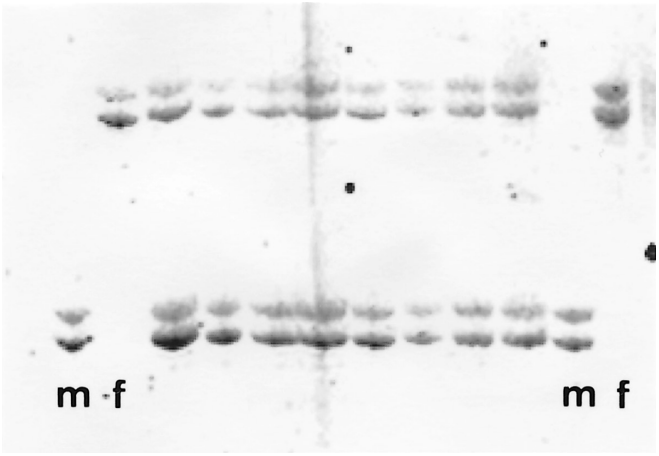

Figure 1. Segregation of microsatellite locus SS10 in an Atlantic salmon half-sib family. M, male parent; F, female parent.

In this work we describe the isolation and characterization of a single trinucleotide locus microsatellite from an Atlantic salmon (*Salmo salar*) cosmid library. We also report the chromosomal location of the microsatellite-containing cosmid clone on the Atlantic salmon chromosome complement.

Materials and Methods

Isolation and Characterization of the Locus Microsatellite

A cosmid genomic library has been constructed in superCosI according to manufacturer's instructions (Stratagene, La Jolla, CA). A $(GAC)_6$ oligonucleotide was kinased with ($\gamma^{32}P$). Positive clones were isolated and DNA extracted by the standard alkali lysis. Cosmid DNA was digested with several restriction enzymes and analyzed by Southern blotting. Positive restriction fragments smaller than 1.3 kb were subcloned into pUC and sequenced with the Sequenase 2.0 sequencing kit (Amersham, Sweden). Clone SS10 was chosen for microsatellite analysis after being mapped by FISH. Two primers flanking the trinucleotide repeat were designed for PCR amplification of this microsatellite (submitted to the EMBL, accession number AJ012206).

FISH

Chromosome obtention. Metaphase chromosomes were obtained from lymphocyte cultures. Two to 3 ml of venous blood was extracted from the dorsal vein of several Atlantic salmon adults and stored in heparinized tubes. Lymphocytes were purified and cultured according to standard procedures. Cultures were incubated at 19°C for 5 days. Six hours before harvesting, colchicine was added to a final concentration of 0.01 μg/ml. Cells were treated with 0.5% KCl and fixed in methanol:acetic acid (3:1). Slides were prepared according to standard procedures.

Probes

The chromosomal location of locus SS10, characterized in this study, was established using as probe the whole microsatellite-containing cosmid clone labeled with biotin 16-dUTP by nick translation according to the manufacturer's recommendations (Roche Diagnostics).

Chromosome slides were pretreated with RNase and pepsin as described by Wiegant et al. (1991). Repetitive sequences were suppressed by prehybridizing 100 ng of the labeled probe with 100 μg of sonicated salmon testes DNA. After overnight hybridization at 37°C, the slides were washed for 10 min at 42°C in 50% formamide 2× SSC and then washed twice for 5 min in 0.1× SSC at 50°C. Detection of signals was performed according to Pendás et al. (1993). Images were obtained using a Zeiss axioscope epifluorescent microscope equipped with a CCD camera (Photometrics).

Microsatellite Analyses

PCR amplifications were carried out using the GeneAmp PCR System 2400 from Perkin-Elmer Cetus, with samples containing approximately 50 ng Atlantic salmon DNA, 10 mM Tris-HCl pH 8.8, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, 20 pmol of each primer, 1 U Dynazyme II DNA polymerase (Finnzymes Oy), and 250 μM dNTP in a final volume of 20 μl. PCRs were performed with an initial denaturing step (5 min at 95°C) followed by 35 cycles consisting of denaturation at 95°C for 20 s, annealing at 57°C for 20 s, and extension at 72°C for 20 s. The final extension was at 72°C for 5 min. PCR products were run on 5.6% acrylamide, 5.6 M urea denaturing gels, and detected by silver staining using the DNA Silver Staining System (Promega). The sizes of allele products were estimated by comparison with pUC sequence reactions.

Mendelian inheritance of this microsatellite locus was tested in two half-sib families. To obtain a first estimate of its variability, 30 wild adults caught in the Esva River (Spain) were analyzed.

Results and Discussion

The detailed sequence analysis of the positive clone SS10 showed that the repeat motif present in this clone was composed of two different interspersed trinucleotide sequences (AAC and GAC), the longest single triple array being seven GAC repeats. According to Jacobson et al. (1993), this sequence can be considered a "cryptic repeat" because the nature of the long tandem repeat is only appreciated when the sequence is categorized into purines and pyrimidines. When this transformation was done to our sequence, we obtained a cryptic repeat consisting of 82 triplets ($cRRY_{(82)}$). In human, mouse, and yeast sequences, cryptic RRY are abundant and, like simple RRY, are nonrandomly distributed with respect to both sequence and location, being the trinucleotides GGC or AGC predominant within human cRRY(i) (Gostout et al. 1993) and preferentially located in coding and 5′ untranslated regions (Ricke et al. 1995). Whether these cryptically simple regions within genes are important for the function of the gene product or represent relatively weakly selected parts of the gene remains unclear.

When the two half-sib Atlantic salmon were analyzed using this cRRY microsatellite, we observed a perfect codominant single-locus Mendelian inheritance (Figure 1). In the sample from the Esva River, seven alleles were detected, ranging from 380 to 456 bp in length. The 434 bp allele showed the highest frequency (0.55), far from the others which showed frequencies of 0.21 (425 bp allele), 0.1 (380 bp allele), 0.06 (413 bp allele), 0.03 (391 and 456 bp alleles), and 0.02 (409 bp allele). The heterozygosity observed for this population was 0.63. In all cases only one or two alleles per individual were observed.

Most of the Atlantic salmon microsatellites characterized in other works are

comprised of two base pair repeat units, usually (GT)n or (GA)n motives. A disadvantage of dinucleotide repeat polymorphisms is that in acrylamide gels, each allele is revealed as several shadow bands that sometimes obscure the position of other allelic fragments, which makes genotyping difficult or impossible: for example, it is difficult to differentiate heterozygotes from homozygotes for alleles differing in length by only two nucleotides.

In this work, and despite the size of the repeat segment in this polymorphic locus (longer than 380 bp), all the alleles at the cRRY microsatellite locus could be identified unambiguously and no stutter bands were observed (Figure 1). This could be due to the complexity of the sequence, which can prevent the substantial polymerase stuttering that is commonly seen when more monotonous tandem repeats [e.g., (GT)n] are amplified by PCR (Gostout et al. 1993). Similar results have been observed by other authors in different species (Edwards et al. 1992; Francisco et al. 1996; Naish 1998; O'Reilly et al. 1996). The unambiguous allele sizing of trinucleotide and tetranucleotide core motives in comparison with dinucleotide core sequences leads us to consider these types of microsatellite loci to be more suitable genetic markers for population analyses.

As expected from the Mendelian monogenic inheritance detected in the two studied families, the FISH of the whole microsatellite-containing cosmid clone shows a single-locus signal in most of the cells analyzed. The signals were located on an interstitial position of an acrocentric chromosome pair (Figure 2). Accordingly we believe that this microsatellite can be used for anchoring the developing genetic and physical map in Atlantic salmon. As previously reported by Lundin et al. (1999) and Martinez et al. (1999), the use of cosmid libraries in Atlantic salmon for isolation and characterization of molecular markers allows the integration of physical and genetic maps and also the identification of the different chromosome pairs.

Looking at the results obtained in this work, we conclude that the isolation of microsatellite markers from cosmid clones is a useful tool for the development of the genetic and physical map in species like fish, with poorly standardized karyotypes.

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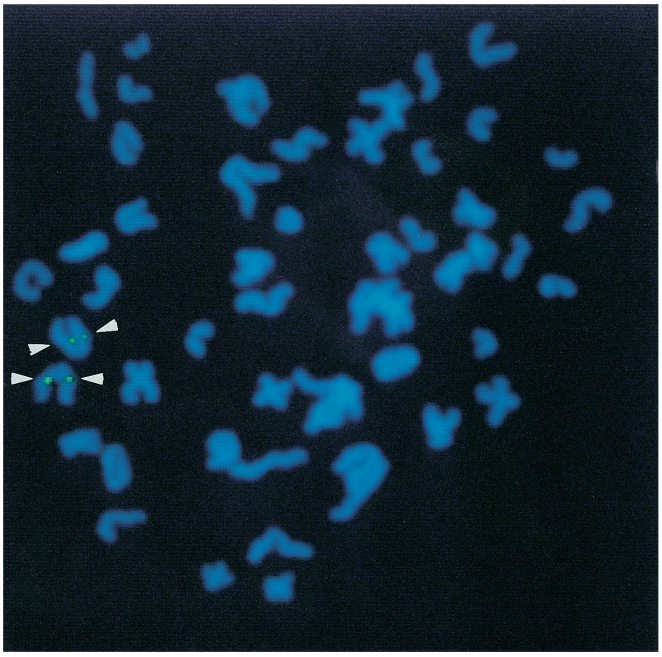

Figure 2. Chromosomal location of the Atlantic salmon SS10 microsatellite locus (arrows).

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A QTL Study of Cattle Behavioral Traits in Embryo Transfer Families

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Two behavioral traits, temperament and habituation, were measured in 130 calves from 17 full-sib families which comprise the Canadian Beef Cattle Reference Herd. Using variance components, heritability was calculated as 0.36 for temperament and 0.46 for habituation. Genotyping of 162 microsatellites at approximately 20 cM intervals allowed the detection of six quantitative trait loci (QTL) for behavior traits on cattle chromosomes 1, 5, 9, 11, 14, 15.

The inheritance of behaviors in domestic animals is of considerable interest to livestock producers, but it has been the focus of relatively few studies. In part, this may be because of the difficulty of assessing or quantifying a behavior for statistical analyses. For example, most studies with cattle rely on a subjective scoring scale to assess temperament during some handling procedure (Dickson et al. 1970; Hearnshaw and Morris 1984; Tulloh 1961; Voisinet et al. 1997). Temperament of an animal can be defined as "an animal's behavioral responses to handling by humans" (Burrow et al. 1997), including its excitatory or inhibitory reactions, level of motor activity, persistent habits, emotionality, alertness, etc. (Hurnik et al. 1995), and as such is not easily quantified. However, certain aspects of temperament such as excitability and the level of motor activity during handling have been quantified (Burrow and Dillon 1997; Stookey et al. 1994) and proven to be persistent over time (Grandin 1993). In addition, these objective measurements have been correlated to at least one physiological response—heart rate (Piller et al. 1999; Waynert et al. 1999). Because selection for certain behaviors is considered to be useful to humans and/or to the animal (Schmutz and Schmutz 1998) it would be beneficial to establish that such behaviors are inherited and therefore could potentially be mapped.

Some studies in humans have been conducted to evaluate relationships between behavior and specific candidate genes, often chosen on the basis of neurochemical properties. Polymorphisms in type 4 dopamine receptor (DRD4) (Ekelund et al. 1999) and in dopamine receptor type 2 (Noble et al. 1998) have been associated with novelty seeking as assessed by the temperament and character inventory (Ekelund et al. 1999). This same assessment was used by Kumarkiri et al. (1999) to conclude that alleles in the serotonin transporter transcriptional control region were associated with cooperativeness. An intronic polymorphism in tryptophan hydroxylase was used as a marker to show it appears to be associated with antagonistic behavior (Manuck et al. 1999).

Quantitative trait loci (QTL) mapping is being used in humans and mice for complex traits, and some of these include behaviors. We report here on a study that attempts to map two behavioral traits using a QTL approach in beef cattle: the response to isolation during handling (which we believe to be a reflection of temperament) and habituation to the handling procedure.

Methods

Animals

A herd of 17 families composed of 130 embryo transfer calves was used in this study. The calves were raised by surrogate mothers or recipient dams, as opposed to their biological mothers. As is typical of cow herds, the sires were not in contact with their calves either. The calves were weaned from their recipient dams at 6 months of age and trucked for approximately 2 h from the ranch where they were born to the University of Saskatchewan Beef Research Station. The newly weaned calves arrived in six groups of a few families each over a period of 4 months. The calves were unloaded from the trucks and group penned until each was individually weighed and measured within the next hour.

Behavior Measurements

Each group was assessed upon arrival at the feedlot and again on a single day when they ranged in age from 8 to 12 months. The difference between the initial score at weaning and this later measurement, we call "habituation," since the animals had been weighed in this same building, and therefore held briefly in this same device, every other week since weaning.

During the behavioral assessment, cattle were moved single file through an indoor handling facility and held individually on an electronic platform scale for 1 min. Solid sliding doors and sides prevented the animal from seeing other cattle. The amount of movement made by the animal during the 1-min test was quantified by an electronic movement measuring device (MMD) attached to the load cells of the weight scale (Stookey et al. 1994). The MMD samples the analogue voltage signal at 122 discrete time intervals per second. Any movement by the animal on the scale causes the signal to fluctuate. A peak is recorded whenever a trend of increasing or decreasing voltage is reversed. The number of peaks recorded is correlated to the amount of movement that can be detected by video analysis (Stookey et al. 1994). We call this response to isolation "temperament," in the sense that agitation and movement during handling can be thought of as a reflection of an animal's temperament.

Heritability Calculation

Heritability was calculated from variance components obtained from analysis of variance (ANOVA) using a nested design with biological dams nested within sires, since each sire was mated to more than one dam and hence had more than one full-sib family.

Genotyping

One hundred and sixty-two microsatellites were selected at approximately 20 cM intervals throughout the genome. We chose microsatellites that had six or more alleles whenever these were available, at approximately 20 cM intervals. Polymerase chain reaction (PCR) was used to genotype all parents and calves from DNA extracted from blood (Buchanan et al. 1994). Genotypes were scored twice independently