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The first radiation hybrid map of a perch-like fish: The gilthead seabream (*Sparus aurata* L)

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Abstract

Among Teleosts, Perciformes are the largest order of fishes and include numerous species of commercial importance. Perciformes also comprise species of primary interest for evolutionary studies and analysis of the sex determination systems and sex chromosome plasticity. Unfortunately, genomics tools and resources for Perciformes remain to be developed. Here, we report the production of a seabream whole-genome radiation hybrid (RH) panel in which quality was ascertained by the construction of a 2-Mb-resolution RH map. The map encompasses 440 markers (288 microsatellites, 82 gene-based markers, and 70 STS) suitable for linkage analysis and comparative mapping studies. Achievement of a RH panel and a whole-genome RH map should contribute to establishing seabream as a fish model among the Perciformes and should be of importance in aquaculture for marker-assisted selection, improvement of growth performance, and disease management. Development of RH maps in a cost-effective manner for other fishes with the described methodology will offer a powerful approach in aquaculture and will provide extended capabilities for comparing vertebrate genome evolution.

Keywords: Perciform; Seabream; RH-panel; Genome map

Ray-finned fishes constitute an extremely diverse taxonomic group that comprises more than 50% of all vertebrate species and more than 95% of all extant fishes belong to the Teleostei [1,2]. Teleost fishes show a very large level of diversity in terms of morphology, physiology, ecology, and behavior. In addition to these remarkable properties and the problem of the maintenance of their global biodiversity, fishes are very important to humans as their consumption represents a sizeable fraction of the human diet [3].

Numerous species have been selected as suitable models to study developmental biology, such as the zebrafish and the medaka, or problems attached to the development of pigment cells and melanoma, as in the case of the platy fish (*Xiphophorus maculatus*). Cichlids, like tilapia, are an important model for the study of speciation. The rainbow trout, for example, is studied not only because of its economical value but also as a model for carcinogenesis, toxicology, and comparative immunology. Sex determination and sex differentiation are also intriguing issues studied in several model and nonmodel species such as medaka and zebrafish but also tilapia or salmonid [4].

During the estimated 160 My of teleost evolution, several rounds of tetraploidization and rediploidization took place that shaped the present genome of the teleosts. In addition to these events, a dramatic process of genome size reduction 20–30 Mya affected some fish lineages like those of the spotted green puffer fish (*Tetraodon nigroviridis*) or torafugu (*Takifugu rubripes*)

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[5,6]. Interestingly it is primarily the compactness of these genomes that led to the sequencing of both genomes, thus providing the first genomic sequences for Percomorpha. Unfortunately, neither of these species is easily amenable to genetic and biological studies because of the difficulties encountered in reproducing and breeding them. Thus it appears clear that for scientific as well as economic reasons, studies of the genomic structure of many fishes in addition to those of medaka [7,8], tetraodon [5], zebrafish [9–11], and fugu [12] would be highly desirable.

Radiation hybrid mapping has proven to be a valuable and powerful approach to producing dense and reliable genome maps. This technology provides resources for genetic studies and comparative genome analysis, assists assembly of deep shotgun sequences, or maximizes light shotgun sequencing, also named survey sequencing, and extracts useful information for genome comparison and synteny analysis [13–16]. However, so far only two fish RH panels have been reported, both for zebrafish [11,17].

Here we report the construction of a seabream RH panel the quality of which was validated by the construction of a map comprising 440 markers. It is anticipated that the method used to construct the RH panel here should be applicable to other teleosts and the seabream RH panel itself a useful tool regarding seabream both as a model system and as a species of great economical value.

Results and discussion

Construction of the RH panel

In the present study, in contrast to the zebrafish RH panels, which were generated from fish cell lines [11,17], we used primary fibroblasts derived from seabream fins—thus avoiding chromosomal rearrangements that could occur in long-term cultured cell lines. Fibroblasts were γ-irradiated at 3000 rad and subsequently fused with hypoxanthine–guanine phosphoribosyltransferase-deficient (HPRT$^-$) hamster cells (CHO) as described under Methods. To assess the genome content of the 170 hybrid cell lines produced, we genotyped 100 seabream markers. This led to the selection of 93 clones on the basis of their overall marker retention rate to constitute the panel. The average marker retention frequency of this panel was estimated at 30% and ranged from 8 to 59% (Fig. 1).

Expansion by cell culture of most of the selected clones was found difficult and culture of each clone was stopped at the stage of confluence of 2 × 75-cm^2 flasks. At this stage, cells from one flask were frozen and DNA was extracted from the cells in the duplicate flask to perform whole genome amplification (WGA) with ϕ29 polymerase [18], which has been shown to be reliable and efficient for amplifying DNA [19–22]. With as little as 10 ng of genomic DNA–about 10^4 cells–one amplification reaction permits amplification of the whole genome from 7500- to 10,000-fold [18]. The amplified DNA is usable directly for genetic testing and for further amplification with ϕ29 DNA polymerase as we observed no loss of markers upon two successive amplifications. Not only does the WGA approach permit the level of amplification to be adapted to the number of markers to be genotyped, and in this way circumvents the difficult problem of cell propagation, but also it represents an advantageous shortcut in term of cost and time. For example, it takes days instead of months to amplify a whole panel of DNA samples and there is practically no limitation to the available amount of DNA. Seabream RH panel characteristics, retention rate of clones, and guidelines for

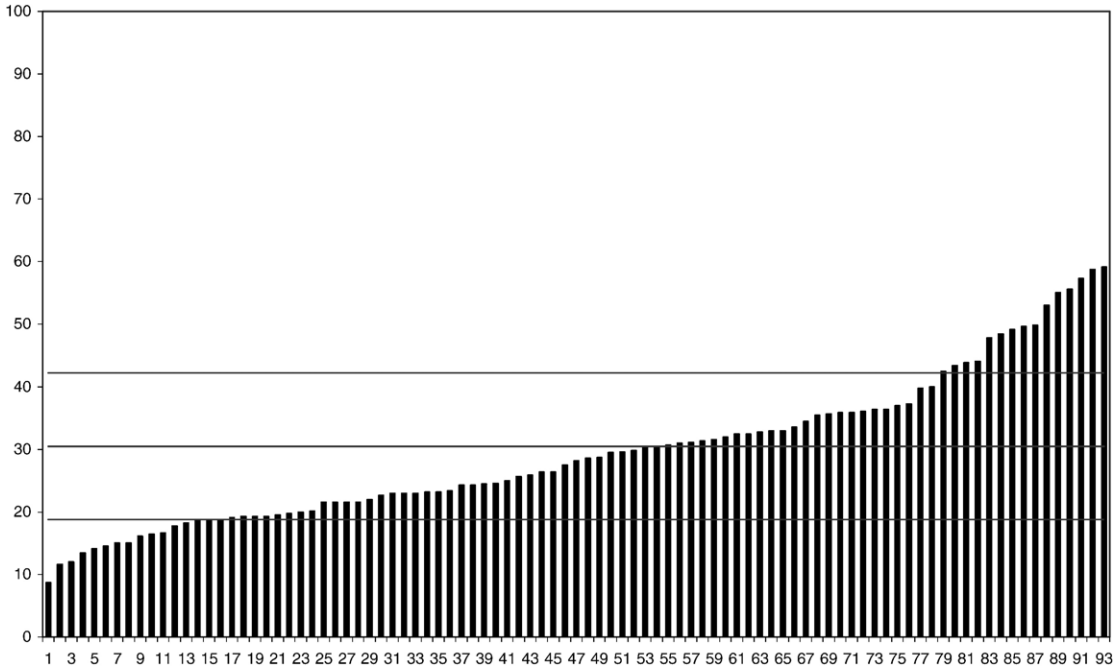


Fig. 1. RH clone retention frequencies. Hybrid cell line retention rate is expressed as the percentage of markers per clone. The average retention of the panel is 30.5% with a standard deviation of 11.75 (*x* axis, clone/cell line number; *y* axis, retention frequency (%)).

receiving the panel can be found in the supplementary online materials (SOM).

Construction of a seabream RH map

This comprehensive seabream RH map consists of 440 markers—82 gene-based markers (representing most genes available in the public databases to date), 288 microsatellites of which 134 have been shown to be polymorphic [23], and 70 sequence-tagged sites (STS). The complete data set was analyzed with the rh_tsp_map2 package, which uses the Traveling Salesman Problem (TSP) approach as specified by the CONCORDE algorithm [24,25]. Linkage radiation hybrid groups (RH groups) were generated initially at a lod score of 5.0 and higher where needed to ensure strong statistical support. Of the 440 markers, 5 markers only (1.2%) remained unlinked to any other marker, suggesting nearly complete genome coverage of the map and an unbiased marker distribution within the 93 hybrid cell lines of the panel. Pair-wise analysis of the RH vectors led to the construction of 28 RH groups (for $n = 24$ chromosomes) ranging from 2 (RH26, RH27, RH28) to 29 markers (RH04), with an average of 15 markers. Multipoint analysis was carried out to order markers within each RH group and to determine their interdistance expressed in centirays (cR_{3000}). The RH map for individual groups ranges in size from 16 to 342 cR_{3000} with an average of 203 cR_{3000} (Table 1, Fig. 2, and SOM). The combined size of all RH groups is 5683 cR_{3000}. Given that the chance of linkage of any new marker is higher than 98% and that the seabream estimated genome size is 800 Mb, 1 cR_{3000} corresponds approximately to 140 kb (800 Mb/5683 cR = 0.141 Mb).

Table 1
RH map characteristics

RH group	No. of markers	No. of positions [a]	Size (cR_{3000})	No. of microsatellites [b]		No. of genes [c]	Tetraodon anchorage [d]		Tetraodon chromosomes [d]
				P	NP/STS		STS	Genes	
1	16	14	342	2	11	3	1	1	7
2	22	21	331	5	12	5	3	2	5
3	24	23	313	8	12	4	5	3	21
4	29	14	310	10	18	1	3	1	9, 18
5	22	21	305	5	13	4	4	3	12
6	18	15	296	5	10	3	3	3	17
7	12	11	286	6	5	1	0	0	4
8	14	14	271	5	7	2	2	0	6
9	23	19	262	7	11	5	2	4	2
10	23	21	261	9	12	2	3	1	1
11	19	18	235	4	6	9	2	5	2, 3, 13
12	19	16	233	5	8	6	5	5	14
13	13	13	221	4	7	2	3	2	2
14	16	14	220	7	8	1	2	0	15
15	20	17	215	4	10	6	2	1	8
16	17	15	206	5	7	5	1	4	11
17	17	15	199	4	8	5	1	3	16
18	13	12	199	4	8	1	4	1	10
19	20	17	194	10	6	4	6	1	13
20	12	11	183	2	8	2	1	2	9
21	15	10	163	5	9	1	1	0	20
22	16	13	119	4	6	6	2	5	3
23	17	13	117	7	7	3	5	1	1
24	8	8	96	3	5	0	0	0	
25	4	4	41	2	2	0	0	0	
26	2	2	29	0	2	0	0	0	
27	2	2	20	0	2	0	0	0	
28	2	2	16	0	1	1	0	0	
Subtotal	435	375	5683	134	221	82	61	48	
				353			109		
					435				
Unlinked	5			1	4				
Total	440	375	5683	358		82	109		

The distribution of the 440 markers within the 28 RH groups along with their main characteristics are presented.

[a] Number of positions on the map occupied by markers. Note that this number can be slightly lower than the actual number of markers (column 2) as some markers can be colocalized.

[b] Microsatellites are detailed as P, polymorphic, or NP/STS, nonpolymorphic or STS.

[c] Number of gene-based markers.

[d] Synteny correspondence with tetraodon, i.e., in RH group 1 there are one STS and one gene marker; both of them are orthologs to tetraodon sequences located in chromosome 7.

In contrast to most mammalian mapping projects and despite several cytogenetic studies [26–29], the seabream karyotype has not yet been standardized; hence, no chromosomal marker localization has been achieved so far. To overcome this lack of information and anchor the RH map to the karyotype, a set of BACs should be used simultaneously for FISH experiments and RH mapping of BAC-end markers [30].

Synteny relationship with other model organism genomes

The ability to map all types of marker on a RH panel provides a valuable and relatively cost-effective means for comparative genomics studies of evolutionarily distantly related species. The 440 seabream marker sequences have been compared with five available genome sequences, human (*Homo sapiens* build 35), mouse (*Mus musculus* build 33), tetraodon (*Te. nigroviridis* V7), fugu (*Ta. rubripes* v.3.0), and zebrafish (*Danio rerio* Zv3), using the BLAT algorithm (http://genome.ucsc.edu/cgi-bin/hgBlat) (see Methods). Of the 440 markers, 162 (36%) have an identified ortholog in tetraodon, 89 (20%) in fugu, 46 (10%) in zebrafish, 31 (7%) in mouse, and 26 (6%) in human (Table 1). Not surprisingly, the number of identified orthologs between seabream and the other species is correlated directly with their phylogenetic proximity [31]. Of the 162 putative orthologs in tetraodon, 67% (109) have a known chromosomal assignment in the tetraodon sequence (Table 1, Fig. 2, and SOM). This result is in agreement with the fraction of the tetraodon sequence (64%) assigned to a given tetraodon chromosome [5].

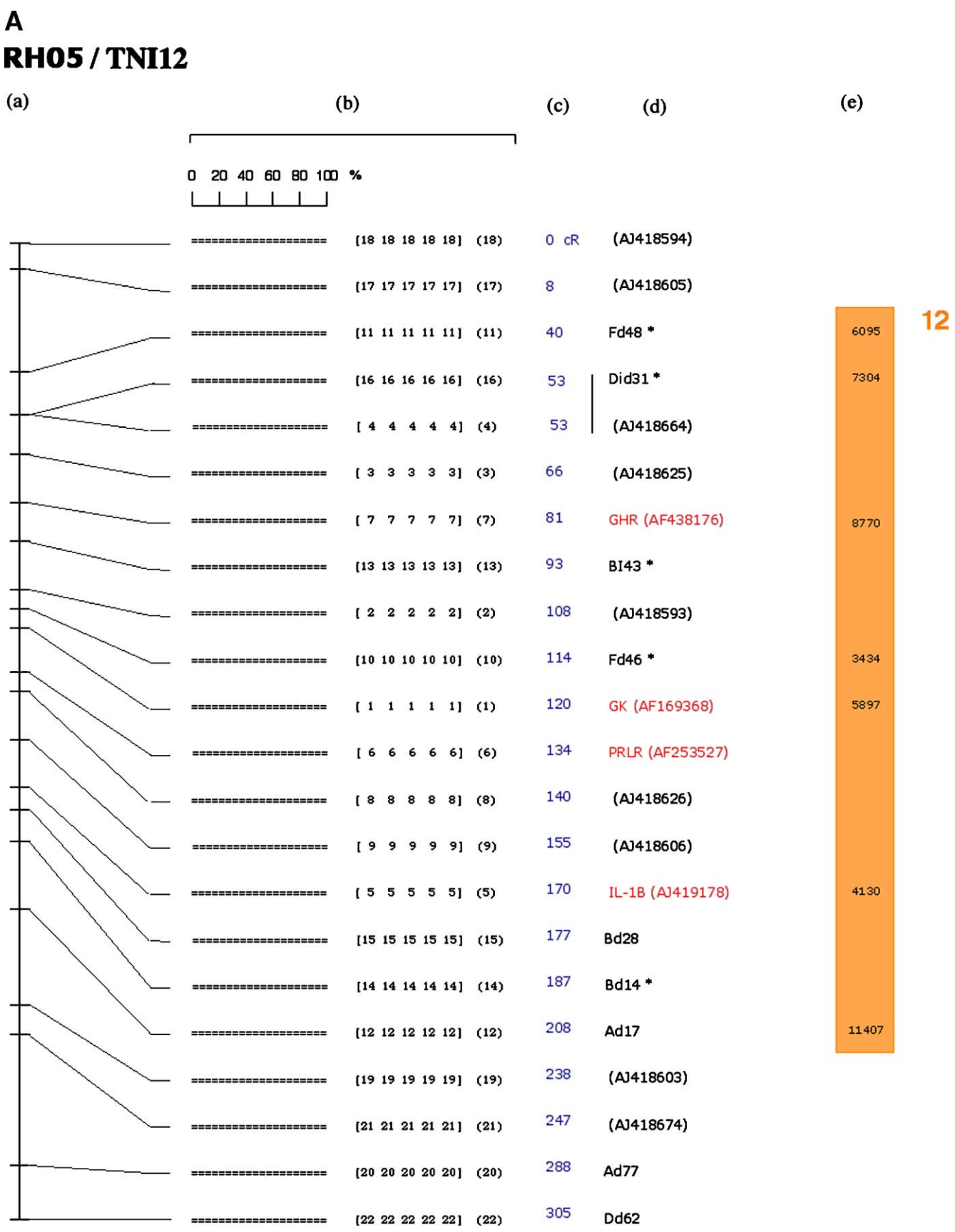
These 109 markers consisting of 48 gene-based sequences, 24 polymorphic microsatellites, and 37 STS are distributed over all seabream RH groups with the exception of the five smallest groups (RH24, 25, 26, 27, and 28) and thus constitute comparative anchors for each of the tetraodon chromosomes, with the exception of tetraodon chromosome 19 (TNI19) (Table 1). With the present set of comparative anchors, 21 conserved syntenic blocks, comprising 2 to 10 markers, were identified when the seabream map and the tetraodon genome sequence were compared, with syntenic breakpoints observed in two RH groups: RH04, corresponding to TNI18 and TNI09, and RH11, corresponding to TNI02, TNI13, and TNI03.

Nine of the 440 seabream marker sequences have a significant match with sequences from the five genomes previously listed. These sequences, which appear to have been particularly well conserved during evolution, correspond to eight genes (ACV-b, ER-α, MLC2, GK, MSTN, MYOD1, MX, GNAT2) and one microsatellite (SAdeca-IMBB01) probably localized close to a functional DNA element. Seabream markers corresponding to these sequences are distributed in different RH groups.

Interestingly we have found a syntenic relationship between the seabream RH23 group and TNI01. It has previously been observed that TNI01 is syntenic to the Xq22–q28 locus of human chromosome X [32,33], which also contains the HPRT gene at position Xq26.1. This suggests in turn that the selective marker HPRT, used for panel construction, might be localized close to the seabream RH23 group. The three smallest groups, RH26, 27, and 28, which contain two markers each, have a high retention value, 75, 94, and 87%, respectively. These groups are probably in the vicinity of the selective marker and one can hypothesize that these groups should merge with RH23 when the number of markers is increased. The results on synteny comparison are consistent with published data reporting that seabream is closely related to fugu and tetraodon [1]. According to the most recent evidence [4–6,34,35] a whole-genome duplication from a prototypic vertebrate genome (n = 12) occurred in the lineage giving rise to teleosts, including seabream and tetraodon, which have 24 and 21 chromosome pairs, respectively. The availability of a powerful seabream RH panel that permits construction of a high-density map will offer a unique opportunity to investigate how these two genomes have evolved after the duplication event, especially with regard to the hermaphroditic status of seabream.

This first map already represents a useful tool for marker-assisted selection for commercially important traits such as disease or stress resistance, which are of notoriously low inheritance. The seabream belongs to the Perciformes (perch-like fish), the largest order of vertebrates, containing over 7000 species, which include several fish families of great importance for genomic studies and fisheries and/or aquaculture (billfish, tunas, basses, porgies, cichlids, …), but none of the current fish model species (zebrafish, medaka, fugu, tetraodon). The development and implementation of a seabream RH map might therefore provide a link between these model species and this large fish assemblage and give this commercially important species model organism status. In addition, the method used to produce this RH panel should be applicable to other fish species and in particular to those of the same phylogenetic group. It is highly probable that for economic reasons few or no other fish genomes will be the object of a deep sequencing effort (7–10× coverage), and only low-pass sequencing at 1–2× coverage (namely survey sequencing) will be carried out. The possibility of constructing fish RH maps backed on survey sequencing should be of great value to navigating the genome of interest and for the analysis of synteny comparison between fish genomes as we have recently shown in the case of the canine genome [15].

Fig. 2. (A and B) Examples of two RH groups (RH05 and RH03) and their syntenic relationship with tetraodon chromosomes (TNI12 and TNI21, respectively). (a) The RH group is symbolized by a vertical bar. The position of each marker is reported along the RH group and symbolized by a horizontal bar. (b) The five RH maps automatically delivered by TSP/CONCORDE are compared. When a marker is present on all five maps at the same position, the horizontal bar has a maximum length, indicating high confidence. When a marker is present at different positions, the horizontal bar is shortened, reflecting a lower confidence level. Numbers in parentheses correspond to the markers as they appear in the consensus map. (c) Distances between RH markers are reported in cR_{3000}. (d) Markers in red are gene based; markers in black correspond to STS and microsatellites; polymorphic microsatellites are noted with an asterisk. Colocalized markers are indicated with a vertical bar to the left of these markers. (e) Colored box to the right of the markers displays tetraodon conserved syntenic block with chromosomal positions as indicated on the tetraodon sequence. XXXX indicates a chromosomal assignment with no known position defined by the genome sequence.


A
RH05 / TNI12
(a)
(b)
(c)
(d)
(e)
0 20 40 60 80 100 %
[18 18 18 18 18] (18) 0 cR (AJ418594)
[17 17 17 17 17] (17) 8 (AJ418605)
[11 11 11 11 11] (11) 40 Fd48 *
[16 16 16 16 16] (16) 53 Did31 *
[4 4 4 4 4] (4) 53 (AJ418664)
[3 3 3 3 3] (3) 66 (AJ418625)
[7 7 7 7 7] (7) 81 GHR (AF438176)
[13 13 13 13 13] (13) 93 BI43 *
[2 2 2 2 2] (2) 108 (AJ418593)
[10 10 10 10 10] (10) 114 Fd46 *
[1 1 1 1 1] (1) 120 GK (AF169368)
[6 6 6 6 6] (6) 134 PRLR (AF253527)
[8 8 8 8 8] (8) 140 (AJ418626)
[9 9 9 9 9] (9) 155 (AJ418606)
[5 5 5 5 5] (5) 170 IL-1B (AJ419178)
[15 15 15 15 15] (15) 177 Bd28
[14 14 14 14 14] (14) 187 Bd14 *
[12 12 12 12 12] (12) 208 Ad17
[19 19 19 19 19] (19) 238 (AJ418603)
[21 21 21 21 21] (21) 247 (AJ418674)
[20 20 20 20 20] (20) 288 Ad77
[22 22 22 22 22] (22) 305 Dd62
6095
7304
8770
3434
5897
4130
11407
12

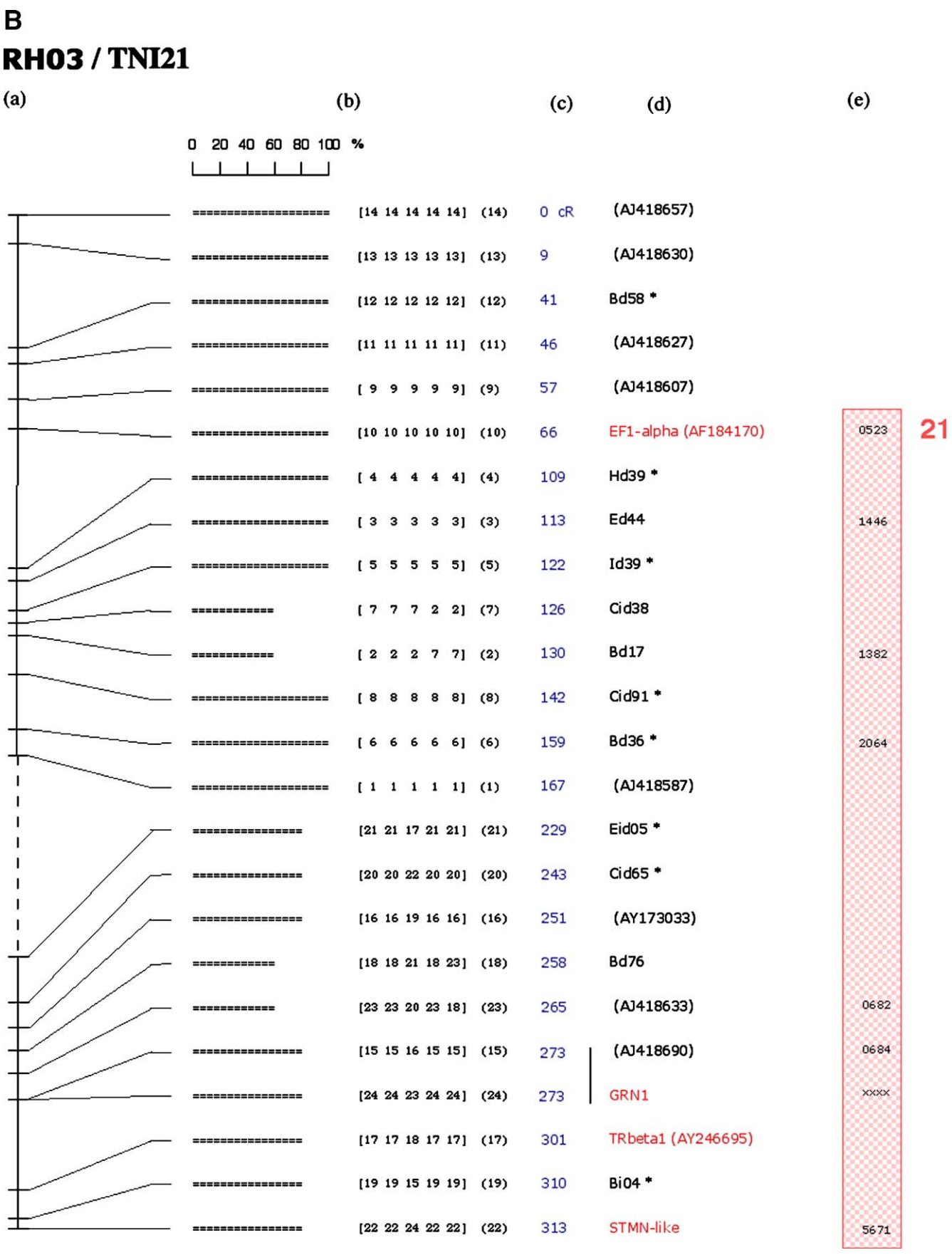


Fig. 2 (*continued*).

Methods

Seabream fibroblast preparation

Fish, 25 g average weight, were kindly provided by the FMD (Ferme Marine du Douhet, Ile d'Oléron, France). The fish were killed by anesthetic overdose, rinsed briefly with 70% ethanol, and washed several times with a washing solution (Leibovitz L-15; 10*Penicillin/Streptomycin/Fungizone; Gibco).

Caudal fins were harvested, cut in small pieces, and subsequently digested three times under constant agitation using trypsin (Gibco). The cell suspension was then centrifuged over a FCS cushion (200*g;* 5 min), washed once, and finally resuspended in culture medium (Leibovitz L-15; 1*Penicillin/Streptomycin/Fungizone; 5% FCS–PAA; FCS-Gold). Cells were counted and seeded at 2×10^4 cells/cm^2 in 75-cm^2 cell culture flasks (Falcon). Cells were maintained at 22°C in a humidified atmosphere. The medium was changed every second day. At confluence (approximately 10 to 15 days later), cells were harvested and frozen until irradiation/fusion experiments.

Generation of radiation hybrid cell lines

A panel of radiation hybrid cell lines was constructed using the methodology previously described [36]. Briefly, seabream fibroblasts were irradiated by a 3000-rad γ-ray exposure. After being counted in presence of trypan blue vital dye, cells were fused with HPRT$^-$ derivative CHO cells (Chinese hamster ovary) in a 1/1 ratio in the presence of polyethylene glycol [37]. Following selection in HAT medium and minimal cell culture expansion (between 3 and 4 weeks), DNA was extracted from individual clones (QIAmp DNA kit, protocol for cultured cells; Qiagen, Hilden, Germany).

DNA amplification

After verification of the presence of seabream DNA and analysis of the retention profile, a total of 93 radiation hybrid cell lines were selected to form the panel. DNA amplification was achieved using WGA with the GenomiPhi DNA amplification kit (Amersham) using the methodology supplied. Briefly, 2 × 10 ng of DNA were separately amplified following the Amersham amplification kit instructions. After the amplification products were pooled, to balance any bias that could have occurred during the amplification process, the DNA was quantified using the PicoGreen methodology (PicoGreen dsDNA Quantitation Kit; Molecular Probes). This led to the production of a stock panel. A working panel was obtained by pooling the DNA samples obtained by four parallel amplification reactions of 10-ng aliquots of the stock panel DNA. This method resulted in 20 μg of DNA, a quantity sufficient for the mapping of several hundred markers, and further working panels could easily be prepared from the stock panel.

Integrity of the WGA procedure was ensured in the following way. We performed by WGA two separate amplification of 10 ng of DNA each of one clone of our canine panel RHDF5000 [36] and analyzed the presence in the amplified DNA of 74 markers that we knew were present and 18 known to be absent from the original DNA. In one of the amplified DNA samples, of the 74 markers, 73 were found present and 1 was absent, and none of the 18 negative markers gave a PCR band in the amplified DNA. In the second amplified DNA sample, the 74 positive markers were present, whereas the 18 negative markers were absent. In another experiment we amplified the DNA of two canine RH cell lines and tested the amplified DNA for the presence of 82 and 88 BAC-end markers. In both cases all the markers except 1 were found in the amplified DNA. Together these control experiments indicated that WGA performed under our conditions is a reliable method.

Marker definition

Oligonucleotide primer pairs, based upon sequences available via GenBank or publications [38], were designed using the Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). A further set of 288 microsatellites was developed in the context of this work. Two microsatellite-enriched genomic libraries [23] were used to isolate repeat-containing sequences from the seabream genome. Accession numbers, characterization, and PCR conditions for all markers are available in the SOM.

Map construction

Genotyping

PCR was performed on 50 ng RH DNA in a final volume of 10 μl containing 0.3 μM each primer, 200 μM dNTPs, 2 mM $MgCl_2$, 50 mM KCl, 10 mM Tris–HCl, and 0.4 U of *Taq*gold polymerase (Applera). Amplifications were carried out with MJ (MJ Research, Cambridge, MA, USA) or GeneAmp 9700 (Applied Biosystems) thermocyclers, with the following PCR program: 1 cycle of 94°C for 8 min; 20 cycles of 94°C for 30 s, 63°C (or 59°C) for 30 s (−0.5°C/cycle), 72°C for 30 s; 15 cycles of 94°C for 30 s, 53°C (or 49°C) for 30 s, 72°C for 30 s. PCR products were analyzed by migration in 2% agarose gels in 0.5× TBE buffer. Gel images were recorded with a high-resolution CCD camera (BioPrint, Vilbert Lourmat, Torcy, France). Results were scored as present, absent, or ambiguous in a semiautomated fashion using an acquisition software. PCR analysis of each of the 440 markers was carried out in single assays, and the results were manually scored as 0 for the absence of a PCR band, 1 for the presence of a PCR band. Faint bands raising concern were scored 2. No vector containing more than three 2's was computed. Moreover, 307 markers taken at random were genotyped in duplicate. We observed the following results: 205 (67%) of these new vectors had no difference from the original, 76 (25%) had one difference, 22 had two differences, and 4 had three differences—a number of discrepancies that we have observed does not alter marker order.

RH computation

The 440-marker RH data set was analyzed using the rh_tsp_map2 package, uses the TSP approach as specified by the CONCORDE computer package (http://www.tsp.gatech.edu/concorde.html) [20]. Using the pairlods_dists program a total of 435 markers could be clustered into RH groups at a lod threshold ≥5.0. Subsequently, multipoint analysis was carried out with rh_tsp_map2 to compute five independent RH maps; three are variants of the maximum likelihood estimate approach and two were constructed using the obligate chromosome breaks approach. The resulting comprehensive maps—they consist of the ordering of all markers within RH groups—were subsequently evaluated to produce a consensus map using a method we previously developed [21]. For markers whose map position was not well supported, genotyping data were systematically reexamined and genotypes repeated as needed. When no erroneous genotypes were observed, the problematic RH group was split into two or more RH groups at lod threshold greater than 6.0. Intermarker distances were determined in centirays (cR_{3000}) with rh_tsp_map2. The complete data set concerning markers can be found in the SOM.

Comparisons with other species sequences

Sequence alignments were performed with the BLAT algorithm. Sequence alignments with a score of 80 or higher (score is defined as twice the matches minus the mismatches minus some sort of gap penalty as defined at http://genome.ucsc.edu/goldenPath/help/blatSpec.html) and a span alignment of 50 bp or higher were retained. These parameters were applied to compare seabream marker sequences with the tetraodon, zebrafish, fugu, mouse, and human genome sequences.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ygeno.2005.11.019.

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