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A genetic linkage map of kelp grouper (*Epinephelus bruneus*) based on microsatellite markers ☆

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ABSTRACT

Kelp grouper (*Epinephelus bruneus*) is an important aquaculture species in Japan, Korea and China. Mariculture production of the species has increased due to its high demand and market price. However, some problems affect kelp grouper aquaculture such as the low growth rate, high mortality due to diseases and low ability to survive the larval stage. To analyze economically important traits, genetic linkage maps are an effective tool. We constructed sex-specific linkage maps of kelp grouper using 222 microsatellite markers. The male map consisted of 23 linkage groups with 161 markers and the female map consisted of 25 linkage groups with 173 markers. The total lengths of the male and female maps were 650.5 cM and 944.4 cM, respectively, and the average intervals were 5.0 cM and 6.7 cM, respectively. The average ratio of recombination between males and females was 1:1.5. Moreover, syntenic sequence comparisons provided basic information of several potential candidate genes affecting organism physiological and biochemical reactions. Based on the linkage map, further quantitative trait loci (QTL) or candidate gene(s) detection can be anticipated to contribute to assist breeding programs of kelp grouper. In addition, by providing basic genome information of kelp grouper, the map provides a first step towards comparative QTL as well as comparative genome analyses with other groupers in the future.

1. Introduction

Kelp grouper (*Epinephelus bruneus*), belonging to the subfamily Epinephelinae in the order Perciformes, occurs in coastal waters of Japan, Korea and China (Heemstra and Randall, 1993). This species is a protogynous hermaphrodite with individuals beginning life as females and subsequently become males (Song et al., 2005). They sexually mature as females at three years old and change sex from females to males at around six years old.

Groupers are highly valued in Asian markets. Among the species, kelp grouper has several advantages for commercial culture such as the quality of their flesh and the reliability of culture techniques. In addition, in this species, research on common problems of groupers such as viral nervous necrosis (VNN) (Nakai et al., 1994) and low survival rate during the larval stage (Teruya and Yoseda, 2006) has enabled progress in mariculture technology. Recently, the manipulation in artificial insemination within one male and one female broodstock has been successful, which makes it possible to build specific family for the construction of genetic linkage map. Therefore, studies of kelp grouper have contributed as a model for other groupers. Genetic linkage maps that contain the markers linked to economically important traits can contribute to improve organisms by marker-assisted selection (MAS) or marker-assisted integration (MAI).

In recent years, genetic linkage maps have been constructed for Perciformes species: European seabass (*Dicentrarchus labrax*) (Chistiakov et al., 2005, 2008), Asian seabass (*Lates calcarifer*) (Wang et al., 2007, 2011a) and gilthead sea bream (*Sparus aurata*) (Franch et al., 2006). In order to facilitate finding the genes controlling economically important traits, QTL mapping in these species has been conducted for better growth (Chatziplis et al., 2007; Massault et al., 2009; Wang et al., 2006, 2008) and cold tolerance (Cnaani et al., 2003). In addition, QTL mapping of disease resistance has successfully been applied in MAS of rainbow trout (Ozaki et al., 2003), Japanese flounder (Fuji

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et al., 2007) and Atlantic salmon (Houston et al., 2008; Moen et al., 2009).

Increased availability of linkage maps constructed from microsatellite markers and other sequence-based markers has led to enable opportunities to accelerate fish genome comparison research. Assembled whole genome sequences of five kinds of model fish and four other kinds of non-model fish species now are available. Blast sequences of mapped marker against these whole genome sequences enable a new insight into identifying prediction of potential candidate genes where QTL have been mapped (Wang et al., 2011a,b).

In order to develop grouper aquaculture, genetic improvement for economically important traits is a powerful tool and construction of a genetic linkage map is necessary to facilitate their use. Therefore, in this study we describe the construction of linkage maps using microsatellite markers with 11 month old kelp grouper.

2. Material and methods

2.1. Reference family and sample collection

A reference family was obtained through the mating between one male and one female grouper. The broodstock groupers were raised in the Ehime Fisheries Research Center, Japan after being captured from the wild. After 11 months from hatching, the parental fish and 90 progenies were used to construct a genetic linkage map. As all the progenies are not mature at this age, the phenotype of sex is not differentiated. DNA was isolated from fin clips using a QuickGene kit (Fujifilm, Japan).

2.2. Genotyping of microsatellite markers

Microsatellite-enriched segments of kelp grouper genome were developed from next generation sequencing (NGS) by GS FLX system (Roche, Switzerland). Other microsatellite sequences were obtained from across species ranging in the subfamily of Epinephelinae according to the NCBI database (Chapman et al., 1999; Dong et al., 2008; Liu et al., 2008; Lo and Yue, 2007; Mokhtar et al., 2011; Ramirez et al., 2006; Renshaw et al., 2010; Rivera et al., 2003; Zeng et al., 2008; Zhao et al., 2009a,b; Zhu et al., 2005). Application software "Websat (www.wsmartins.net/websat)" was used for designing primer pairs. Primers were developed using the default settings, considering the product size from 100 to 250 bp. Marker polymorphisms were tested using the parental fish and four progenies.

Forward primers for each pair were labeled with tetrachloro-6-carboxy-fluorescine (TET) fluorescent dye at the 5′-end. PCR for genotyping was performed in 11 μl volumes containing 0.05 pmol/μl of forward primer, 0.5 pmol/μl of reverse primer, 1× reaction buffer, 2.0 mM $MgCl_2$, 0.2 mM dNTPs, 1% BSA, 0.025 U of Taq polymerase (Takara: Ex-Taq, Japan) and 25 ng of genomic DNA. Cycle amplification was performed in MJ PTC-100 (Bio-Rad, USA) for 5 min at 95 °C as an initial denaturation, 36 cycles of 30 s at 95 °C, 1 min at an annealing temperature (56 °C), 1 min at 72 °C and 10 min at 72 °C for final extension. Amplification products were mixed with an equal volume of loading buffer (98% formaldehyde, 10 mM EDTA and 0.05% bromophenol blue), heated for 10 min at 95 °C and then immediately cooled on ice. The mixture was loaded onto 6% Page-plus gel (Amresco, OH, USA) containing 7 M urea and 0.5× TBE buffer. Electrophoresis was performed in 0.5× TBE buffer at 1800 V constant voltage for 1.5 h. After electrophoresis, the gel was scanned and imaged using an FMBIO III Multi-View fluorescence image analyzer (Hitachi-soft, Japan).

2.3. Genome size estimation

Genome length was estimated in sex-specific maps using two methods. (1) G_{e1} was calculated to account for chromosome ends by adding 2 times the average framework marker spacing to the length of each linkage group, where framework marker was denoted as the marker providing statistical support of marker order and spacing in genetic linkage map (Fishman et al., 2001). (2) G_{e2} was calculated by multiplying the length of each genetic linkage group by $(m + 1)/(m - 1)$, where m means the number of framework markers on the linkage groups (Chakravarti et al., 1991). The estimated genome length (G_e) for each sex was used as an average of the two estimates.

2.4. Linkage analysis

Genotype scoring was performed by using LINKMFEX ver. 2.3 application package (Danzmann, 2006). The application can separate the alleles originated from the male or female parent and check for the accuracy of genotypes in their progeny. Linkage analysis was performed using genotype data converted to a backcross format. As grandparent genotypes were unknown, pairwise analyses were performed, and markers were sorted in linkage groups at LOD threshold of 4.0. Linkage phases were determined retrospectively by examining the assortment of alleles among linked markers. Then the allele was tested for goodness-of-fit for Mendelian segregation distortion using χ^2-analysis. Also the order of the marker loci was confirmed to be correctly positioned, and was checked by double recombination events with the software application program in Map Manager QTX (Manly et al., 2001). Graphic representations of linkage groups were generated with MAPCHART version 2.1 (Voorrips, 2002) using raw recombination fractions as estimates of map distances (Fig. 1-1 to 1-24). In addition, a consensus linkage map was constructed by JoinMap version 4 (Van Ooijen, 2006). Module of combined groups for map integration was used to integrate the sex-specific linkage maps.

2.5. Prediction of potential candidate genes

The microsatellite markers located in the genetic linkage map were used to identify potential candidate genes. We did a sequence similarity search of the flanking sequences of these markers against the whole genome sequences of three-spined stickleback, medaka and Nile tilapia. BLASTN searches were carried out from http://www.ensembl.org/Multi/blastview. Soft masked whole genome DNA database was added before sequence alignment. Only the sequence matches having a minimum alignment length over 50 bp and hits with $e < 10^{-5}$ were used to detect possible candidate genes.

3. Results

3.1. Microsatellite markers

A total of 645 microsatellite primers were designed. Of them, 285 microsatellite markers (Ebr00001FRA to Ebr00014FRA, Ebr00016FRA to Ebr00018FRA, Ebr00020FRA, Ebr00021FRA, Ebr00023FRA to Ebr00038FRA, Ebr00040FRA to Ebr00057FRA, Ebr00059FRA to Ebr00175FRA, Ebr00177FRA to Ebr00228FRA, Ebr00230FRA to Ebr00287FRA, Ebr00289FRA to Ebr00293FRA), were newly developed in the present study. Sequence data of these microsatellites have been deposited in the DDBJ database under the accession series from AB755818 to AB756102. The remaining 360 microsatellite markers (EawSTR2DB–PleSTR385DB) were designed based on cross species sequences collected from the NCBI database. Among 285 designed primer pairs by next generation sequencing (NGS), 146 exhibited polymorphisms in the mapping family. Furthermore, 76 of 360 loci from cross species also exhibited polymorphisms. A total of 222 primer pairs were informative to the reference family. A list of microsatellite markers used for mapping is presented in Supplementary data file 1.

3.2. Linkage map

The male linkage map contained 161 markers distributed in 23 linkage groups (EBR1M–EBR23M) (Fig. 1). The estimated total genome size

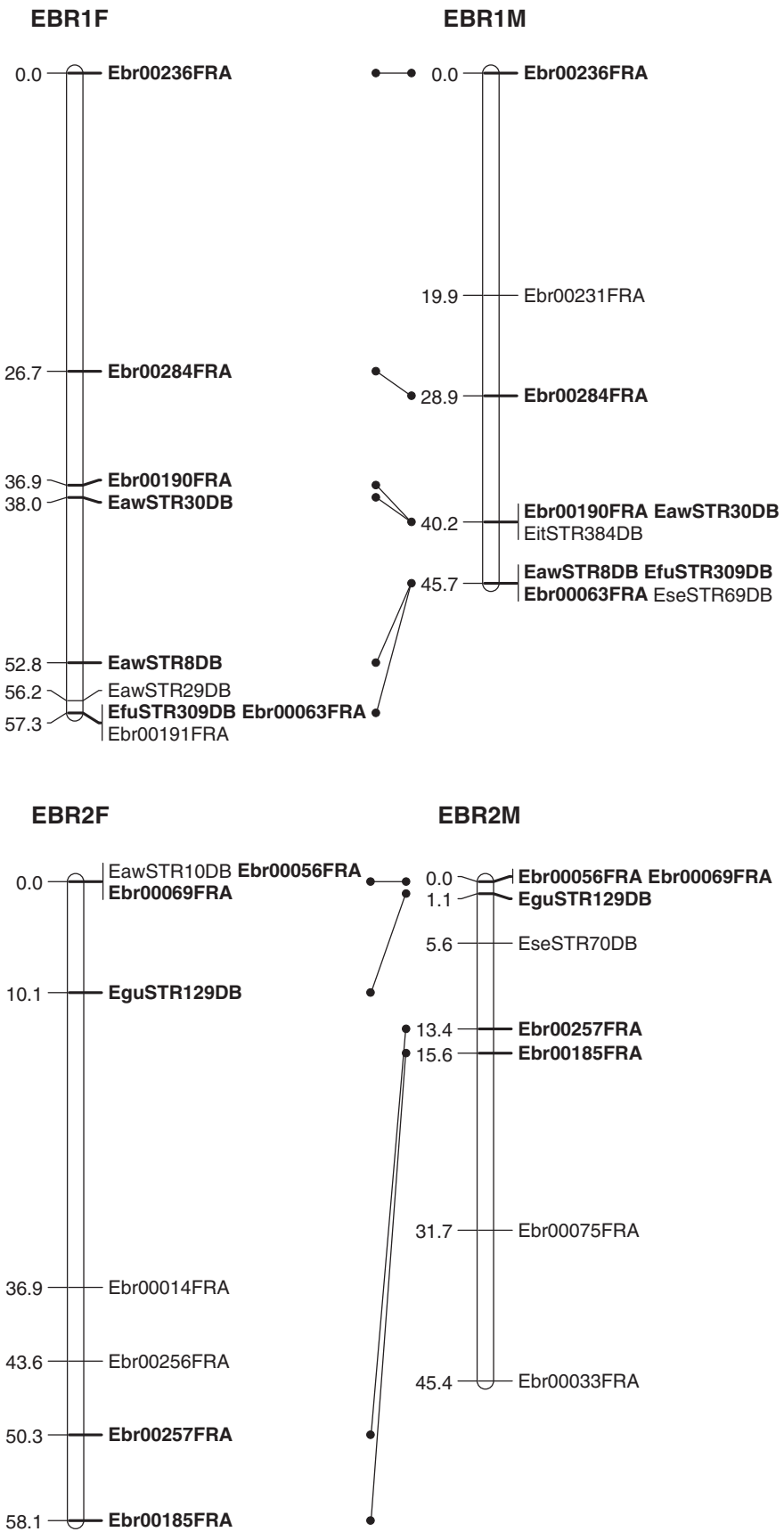


Fig. 1. (Fig. 1-1 to 1-24). Kelp grouper female (left) and male (right) maps, linkage groups EBR1–EBR25. Bold letters indicate the co-segregation microsatellite loci between female and male maps. Map distances calculated between markers are expressed in Kosambi function (cM).

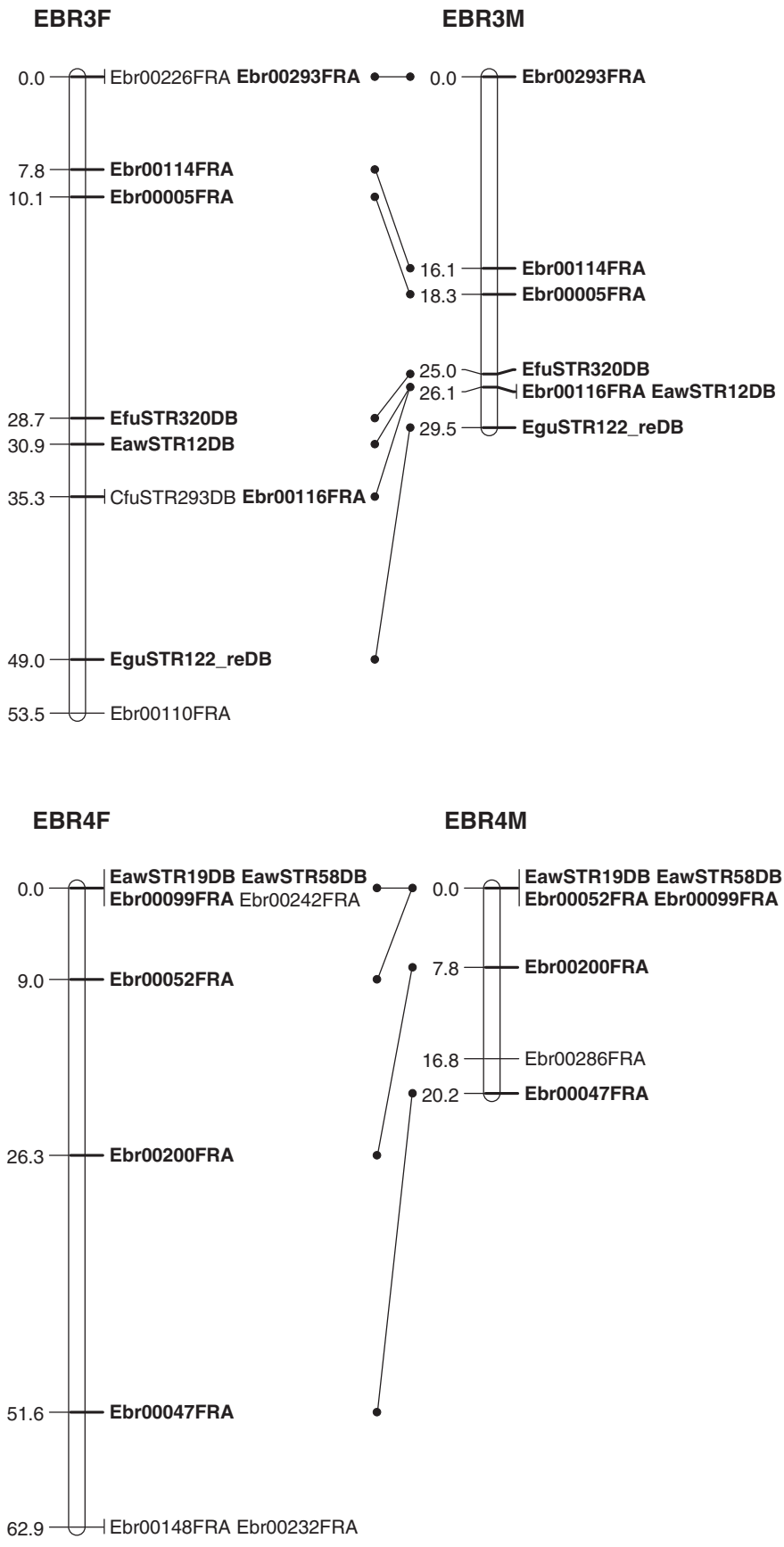


Fig. 1 (*continued*).

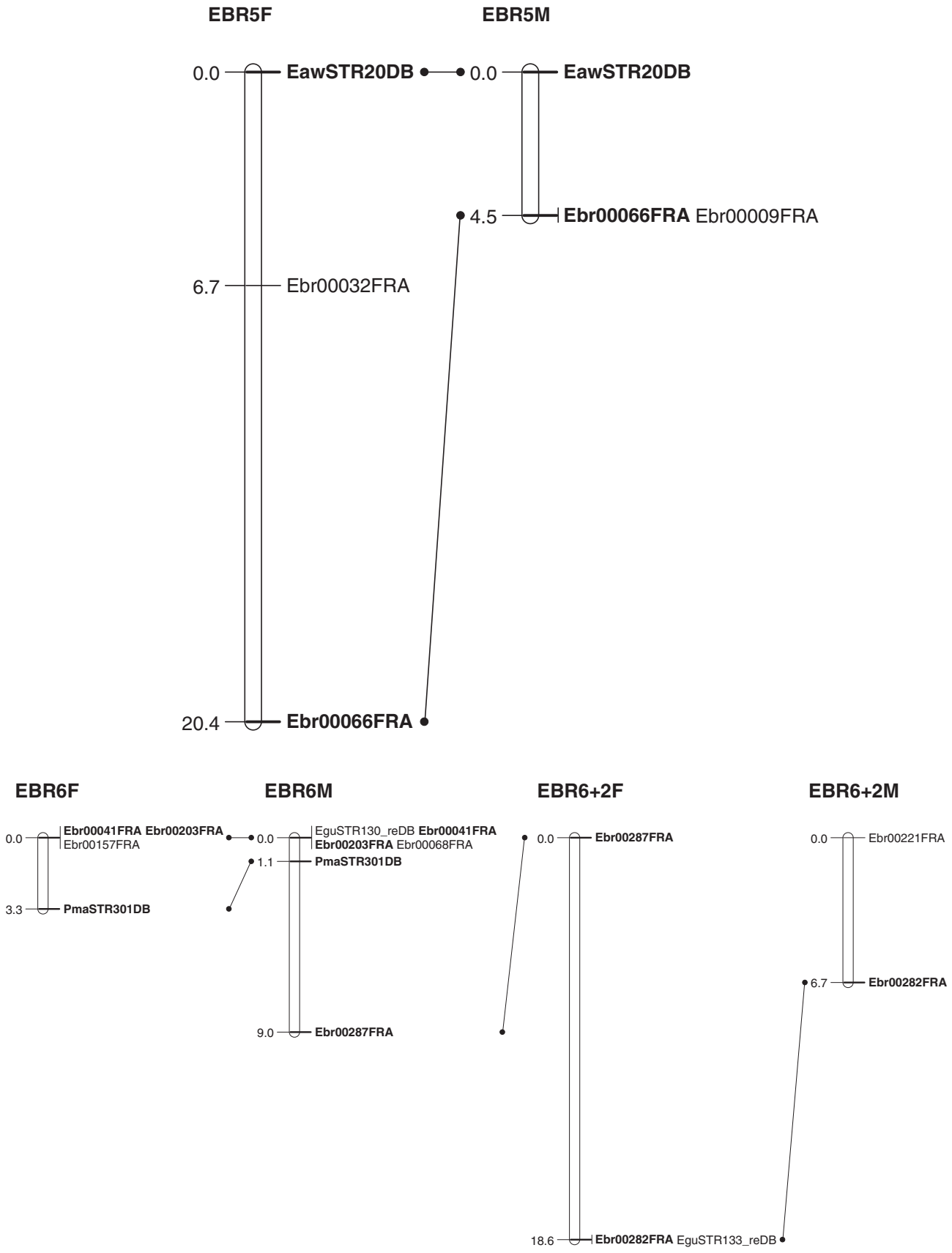


Fig. 1 (*continued*).

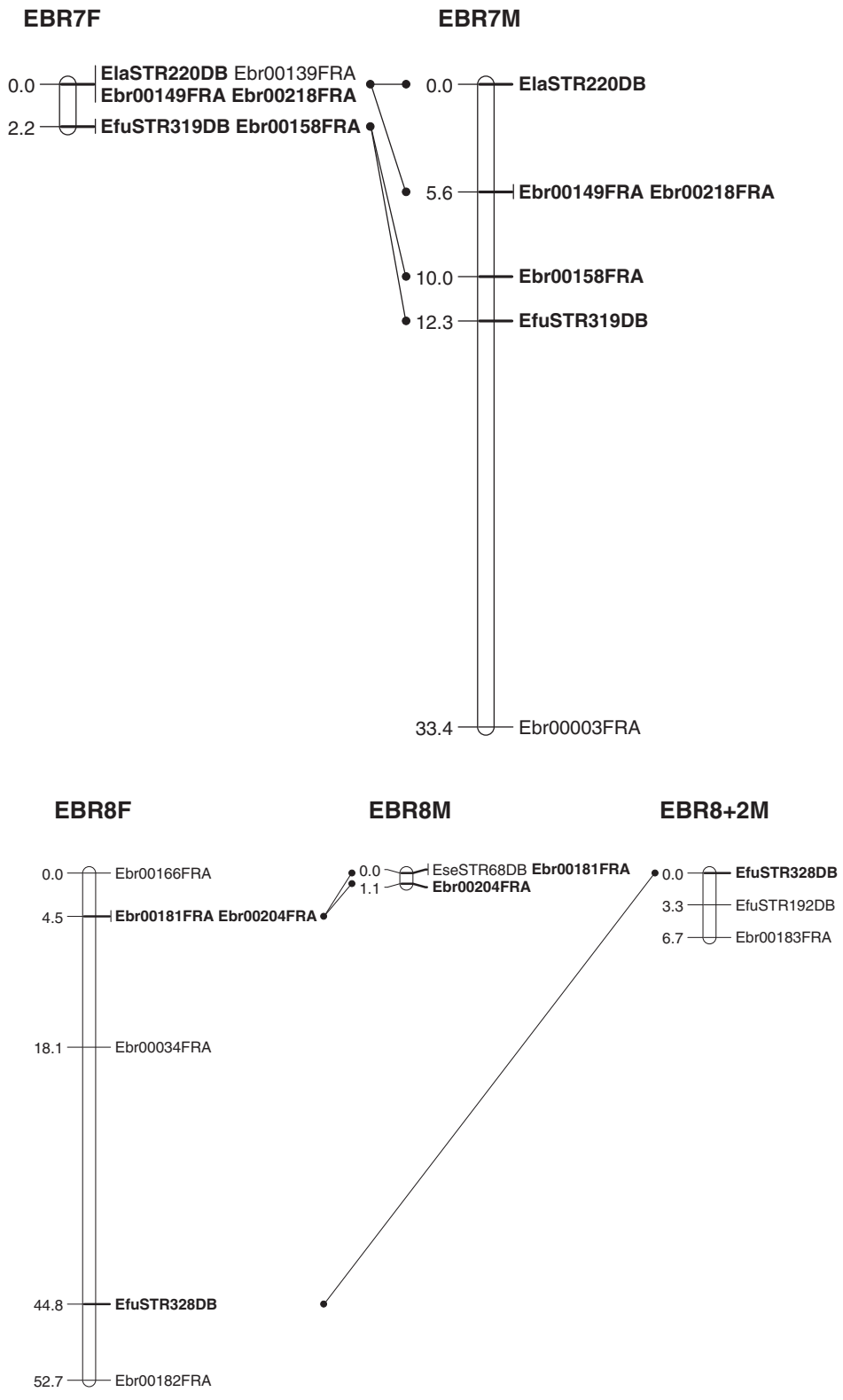


Fig. 1 (*continued*).

of the male map was 650.5 cM and the length of each group ranged from 0 to 58.5 cM. The number of microsatellite markers per linkage group varied from 2 to 10 markers with an average of 7 markers. The female linkage map contained 173 markers distributed in 25 linkage groups (EBR1F–EBR25F) (Fig. 1). The number of markers per linkage group varied from 2 to 11 with an average of 7 markers. The estimated total genome size of the female map was 944.4 cM and the length of each group arranged from 0 to 62.9 cM. The framework marker interval in each group was estimated based on the length between clusters due to some markers formed the clusters. The male and female linkage maps consisted of 122 and 128 clusters, respectively, and the average intervals were 5.0 cM and 6.7 cM, respectively (Table 1). The consensus genetic linkage map as a reference is shown in Supplementary data file 2.

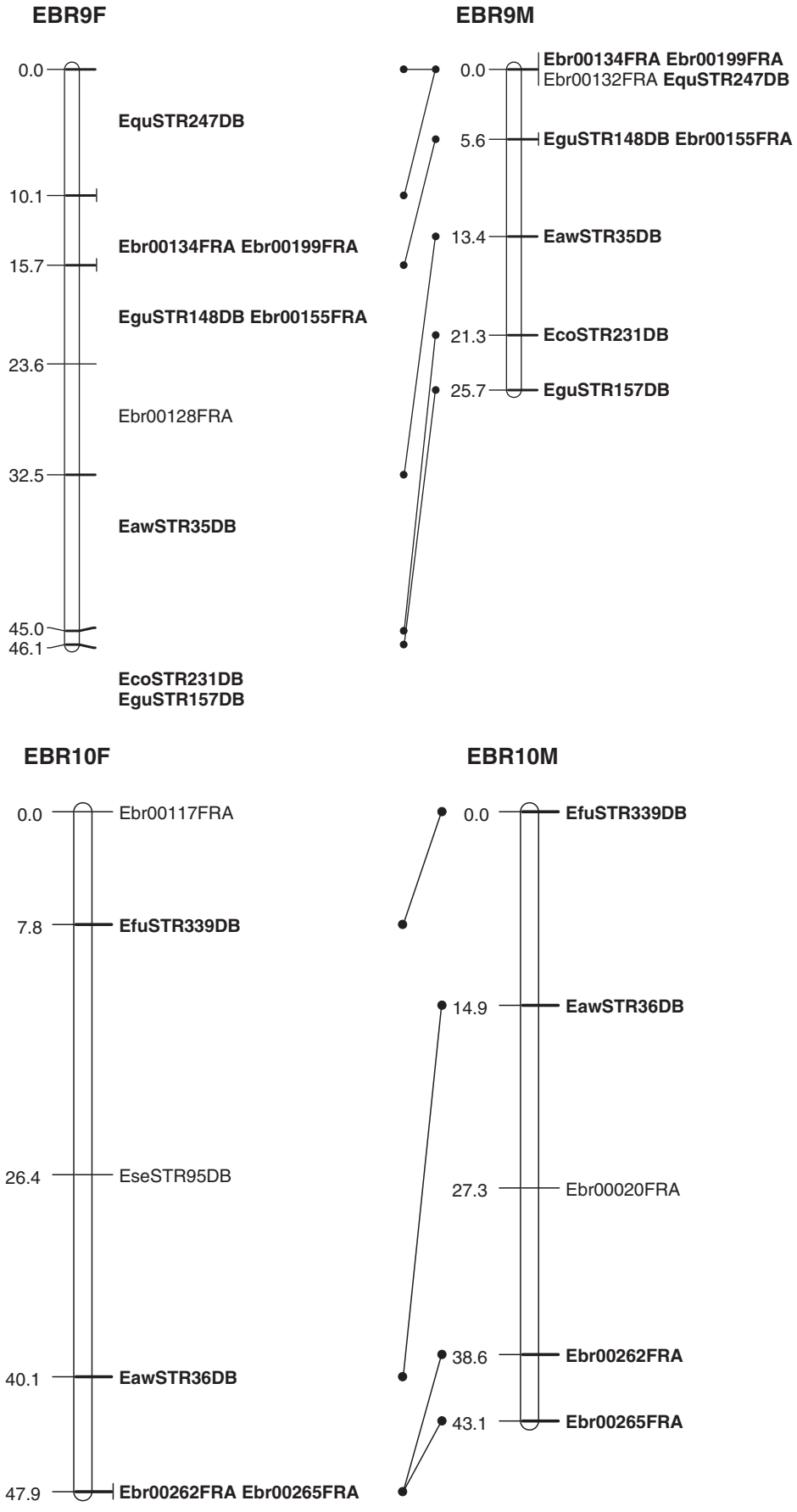
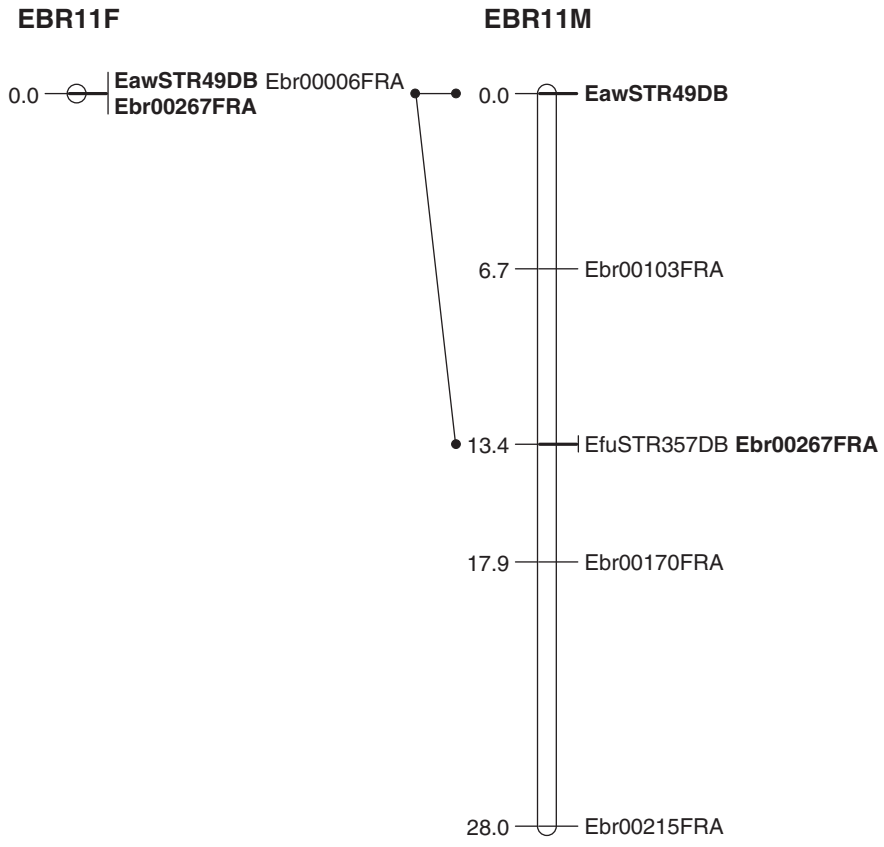


Fig. 1 (*continued*).



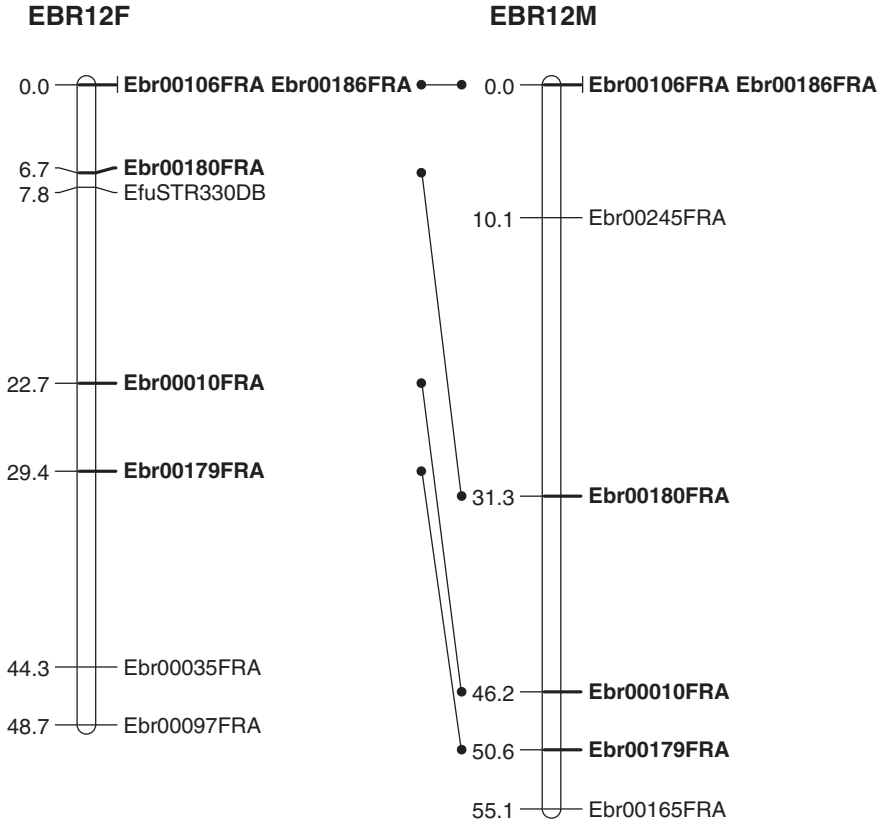


Fig. 1 (*continued*).

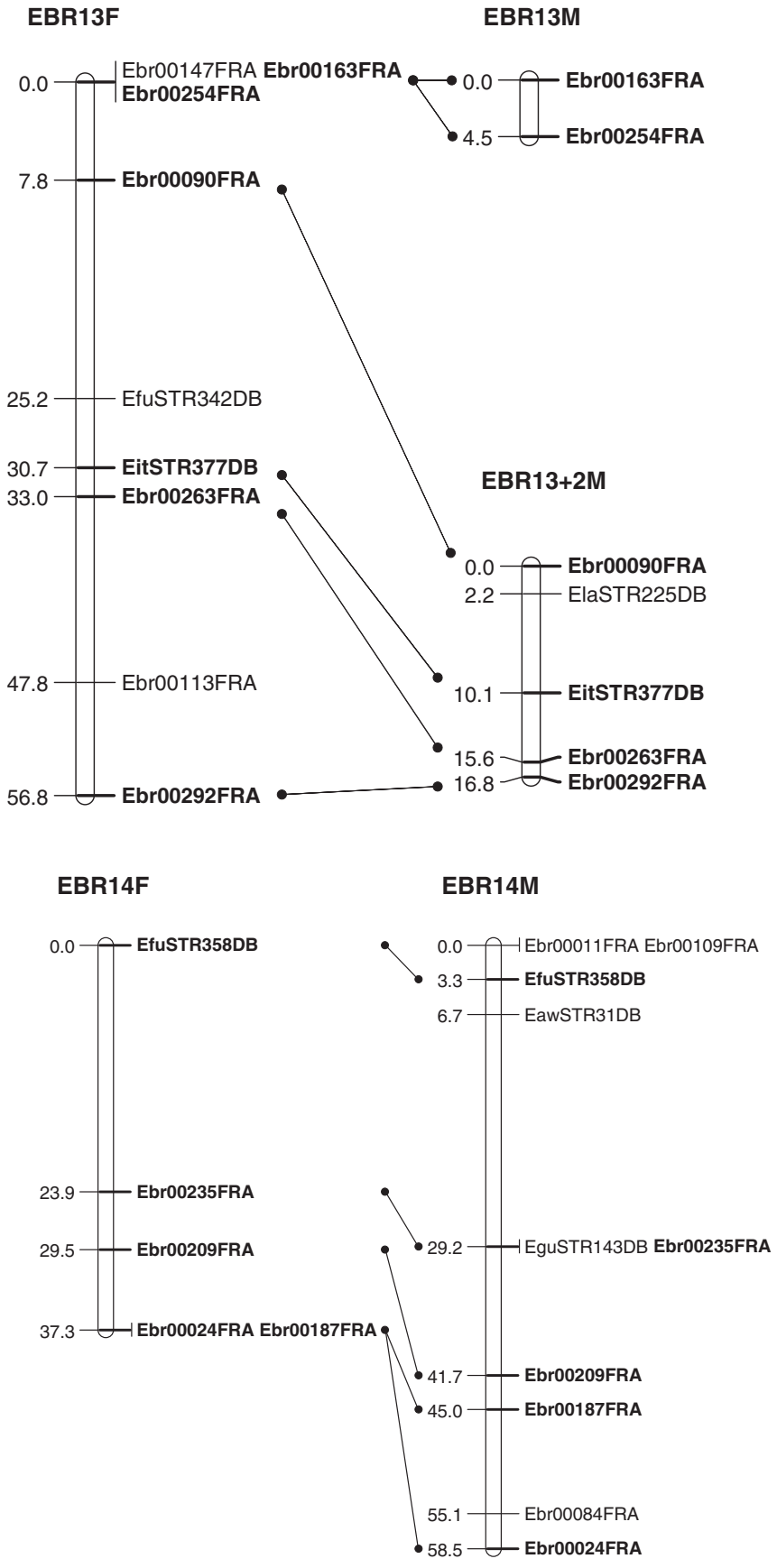


Fig. 1 (*continued*).

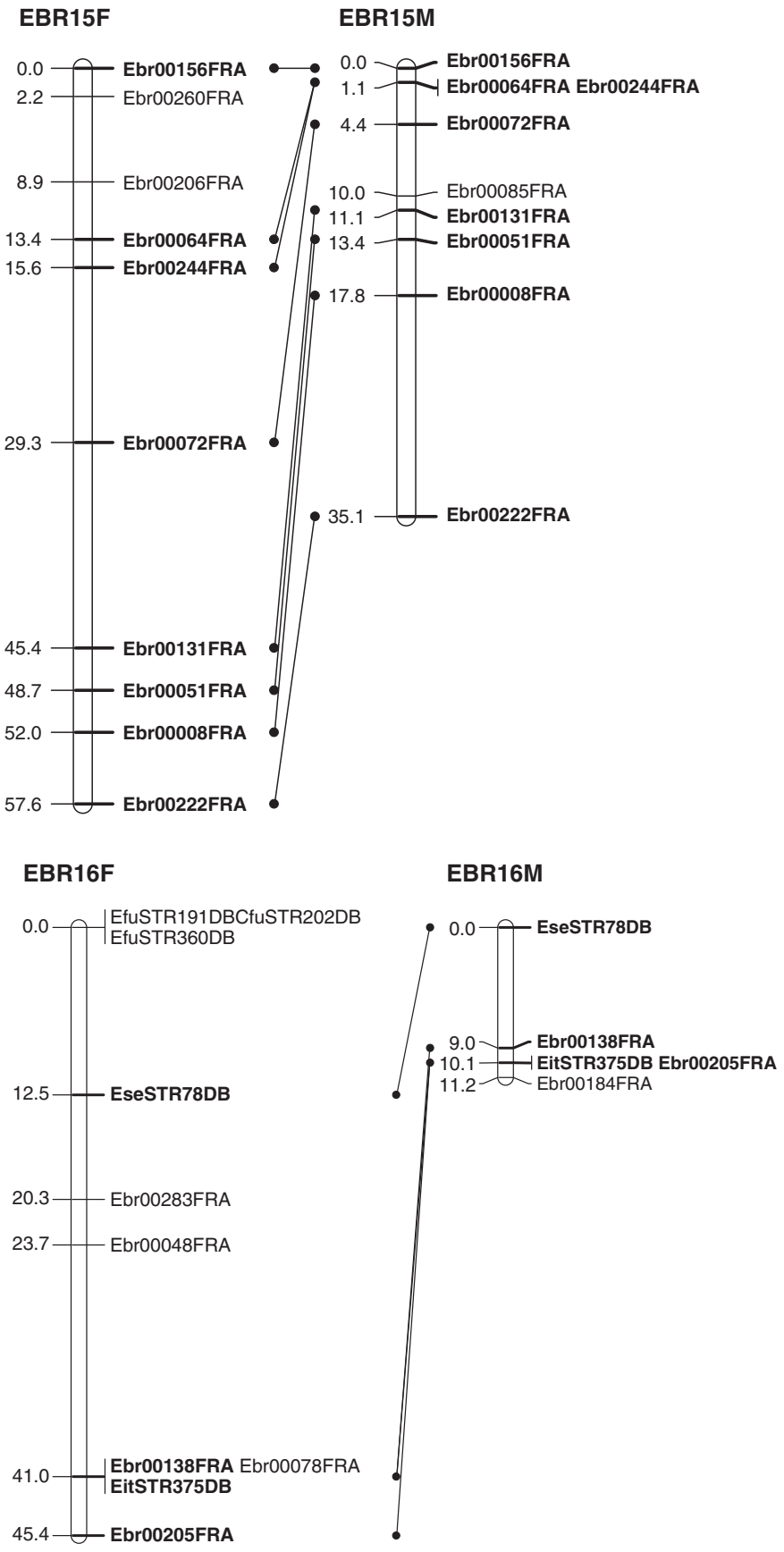


Fig. 1 (*continued*).

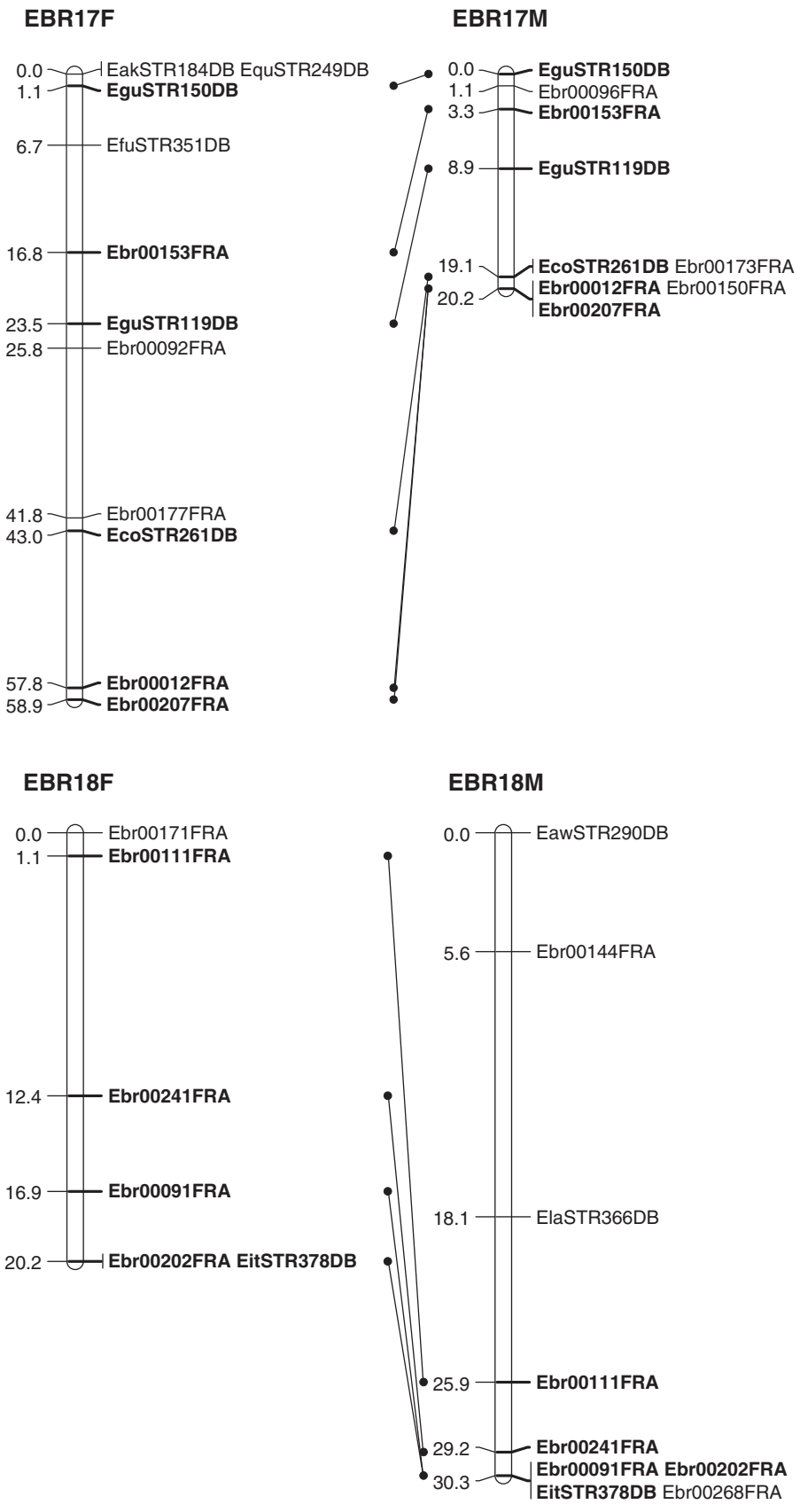
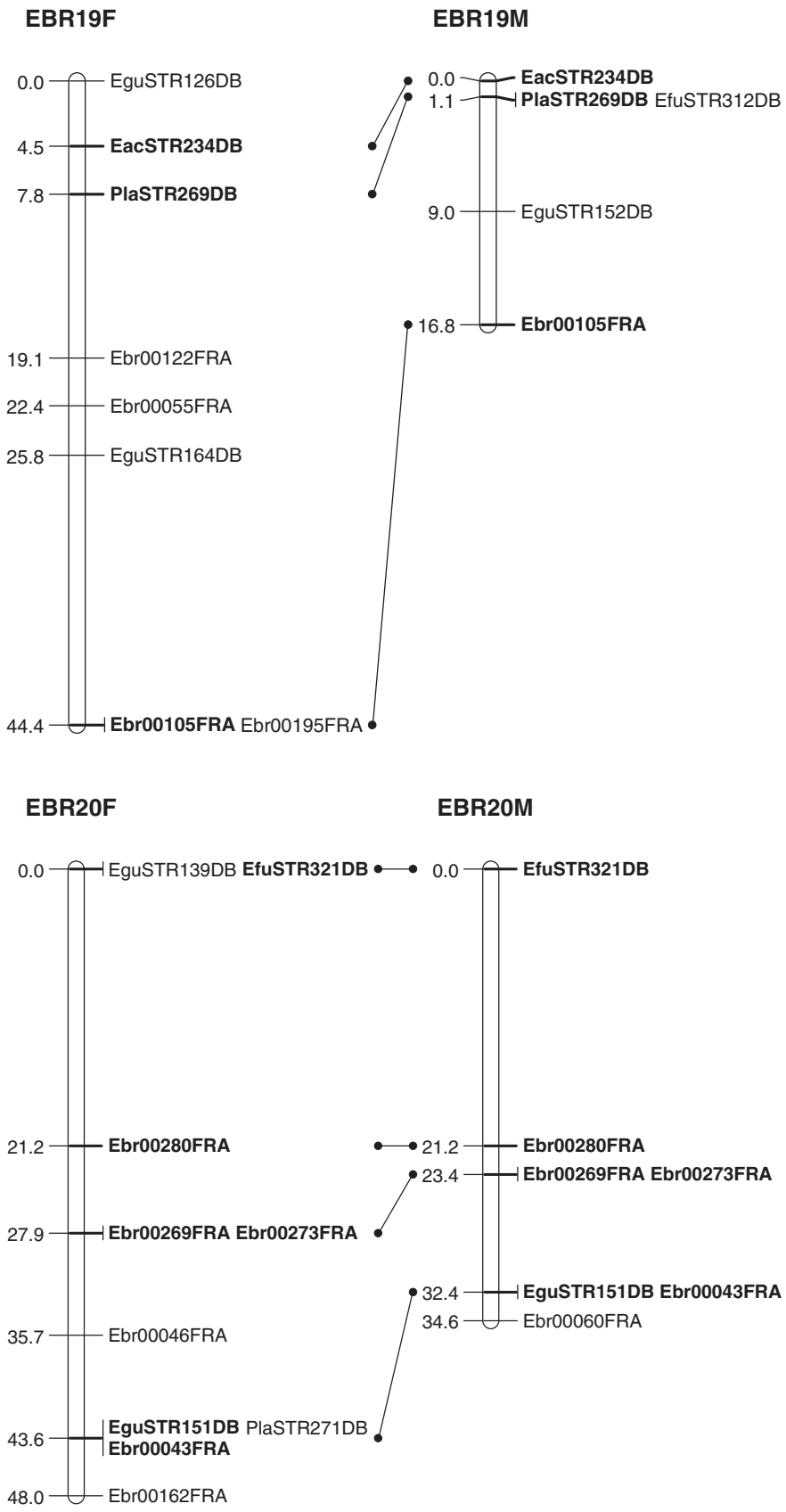


Fig. 1 (*continued*).



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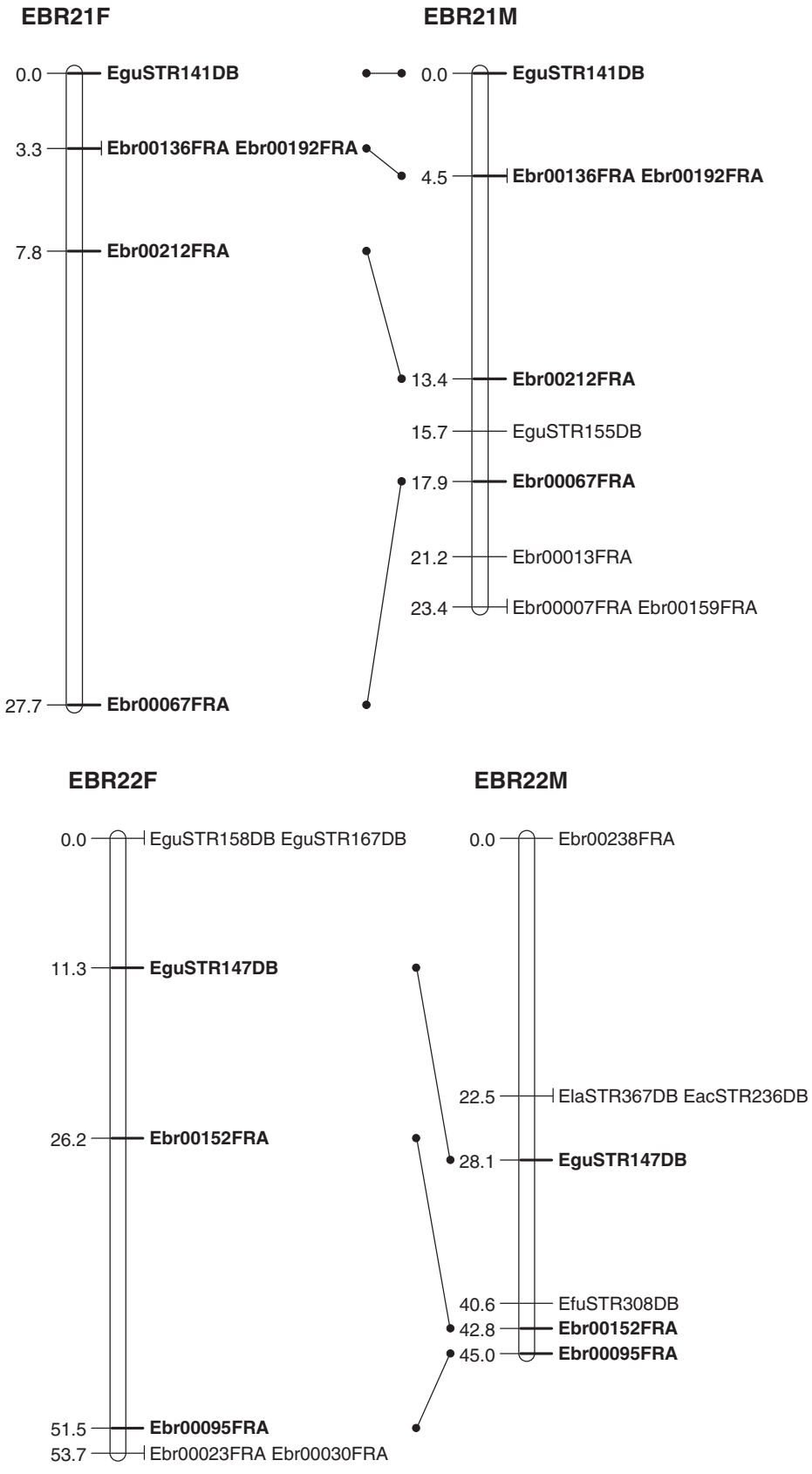

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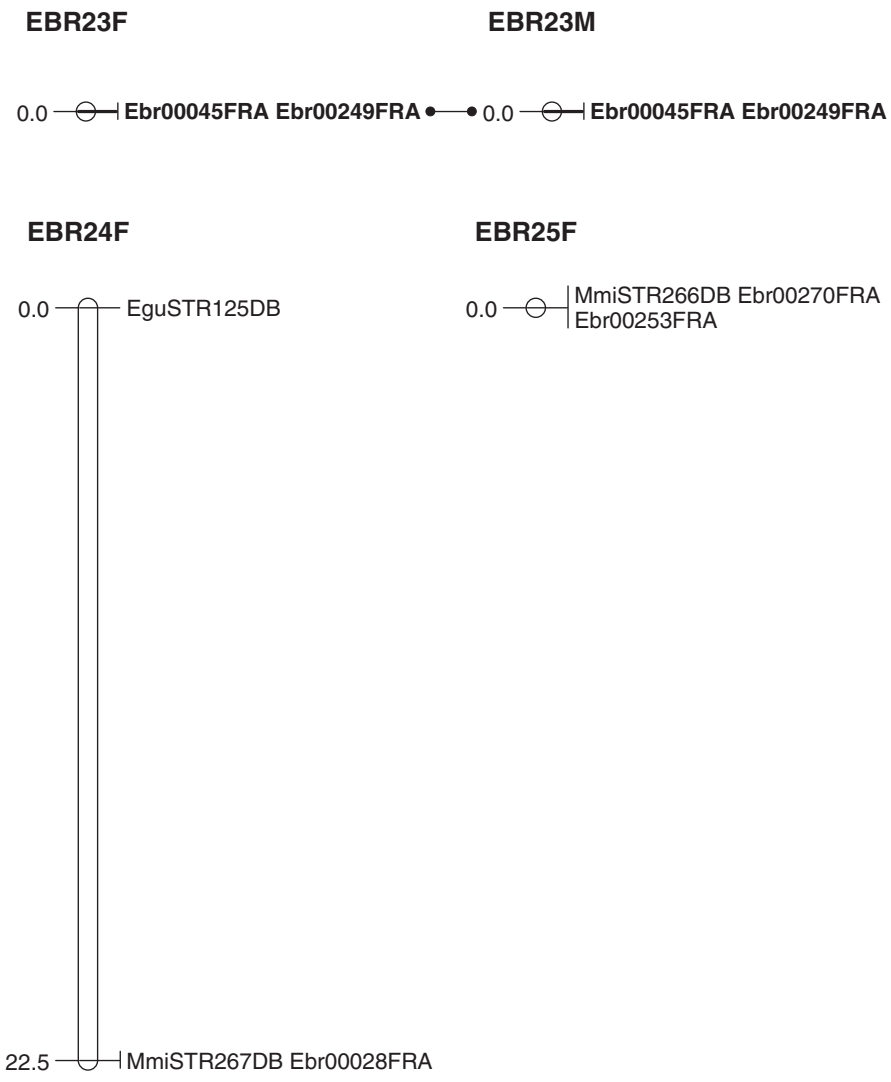

Fig. 1 (*continued*).

3.3. Differences in recombination rate between males and females

Co-segregation markers were used to estimate the recombination rate between the sexes. Twenty-three linkage groups (EBR1–EBR23) in male and female maps shared at least 2 microsatellite loci and could be used to calculate the recombination rate among adjacently paired markers. When taking account all the common intervals, the total length of genetic distance of co-segregation markers in all 23 linkage groups was 493.1 cM for the male map and 762.6 cM for the female map (Table 2). Thus, the average ratio of recombination between sexes was 1:1.5. Female linkage groups had a higher recombination rate than male linkage groups except for linkage groups of EBR7, 8, 10, 11, 12 and 14.

3.4. Estimated genome length

Estimated genome lengths based on the two methods were very similar. For males they were 952.7 cM (G_{e1}) and 967.1 cM (G_{e2}) with an average of 959.9 cM (G_e), and for females they were 1402.8 cM (G_{e1}) and 1406.3 cM (G_{e2}) with an average of 1404.6 cM (G_e). Based on the estimation of map lengths, the genome coverage of the male and female maps (C_f) were 67.8% and 67.2%, respectively. The female map was 1.5 times longer than the male map. A summary of the genetic linkage maps of kelp grouper is shown in Table 3.

3.5. Identification of potential candidate genes

Similarity search of microsatellite markers located in the genetic linkage map against the whole genome of three-spined stickleback, medaka and Nile tilapia revealed several potential candidate genes. A list of potential candidate genes is presented in Supplementary data file 3. In summary, a number of 51 potential candidate genes showing similarity to the flanking sequences of the markers are located in kelp grouper linkage map. It included a number of 37 genes showing similarity to the flanking sequences of the markers located in male linkage map, and a number of 35 genes showing similarity to the flanking sequences of the markers are located in female linkage map. Among these microsatellite markers, Ebr00085FRA located in the linkage group 15, which maker sequence had a significant similarity with the ELTD1 gene responsible for immune effect. The gene is located at around 12.0 Mb in the chromosome 8 of three-spined stickleback and at the position of scaffold GL831309.1 in the genome of Nile tilapia. EawSTR58DB in linkage group 4 showed similarity to the Robo3 gene located at the position of 11.9 Mb of chromosome 7 in three-spined stickleback and at GL831214.1 in Nile tilapia, which had functions in the development of gonadotropin-releasing hormone (GnRH) neuronal system (Cariboni et al., 2012). Moreover, at least nine sequences of the markers (Ebr00128FRA, Ebr00132FRA, Ebr00106FRA, Ebr00253FRA, EseSTR68DB, EfuSTR330DB, EfuSTR342DB, EfuSTR312DB and EacSTR236DB) showed

Table 1
Information on genetic linkage maps of kelp grouper.

LG	Male				LG	Female			
	No. of markers		Total length (cM)	Interval length (cM)		No. of markers		Total length (cM)	Interval length (cM)
	Mapped markers	Framework markers				Mapped markers	Framework markers		
EBR1M	10	5	45.7	9.1	EBR1F	9	7	57.3	8.2
EBR2M	8	7	45.4	6.5	EBR2F	8	6	58.1	9.7
EBR3M	7	6	29.5	4.9	EBR3F	10	8	53.5	6.7
EBR4M	7	4	20.2	5.0	EBR4F	9	5	62.9	12.6
EBR5M	3	2	4.5	2.2	EBR5F	3	3	20.4	6.8
EBR6M	6	3	9.0	3.0	EBR6F	4	2	3.3	1.7
EBR6 + 2 M	2	2	6.7	3.4	EBR6 + 2 F	3	2	18.6	9.3
EBR7M	6	5	33.4	6.7	EBR7F	6	2	2.2	1.1
EBR8M	3	2	1.1	0.6	EBR8F	6	5	52.7	10.5
EBR8 + 2 M	3	3	6.7	2.2	–				
EBR9M	9	5	25.7	5.1	EBR9F	9	7	46.1	6.6
EBR10M	5	5	43.1	8.6	EBR10F	6	5	47.9	9.6
EBR11M	6	5	28.0	5.6	EBR11F	3	1	0.0	0.0
EBR12M	7	6	55.1	9.2	EBR12F	8	7	48.7	7.0
EBR13M	2	2	4.5	2.2	EBR13F	9	7	56.8	8.1
EBR13 + 2 M	5	5	16.8	3.4	–				
EBR14M	10	8	58.5	7.3	EBR14F	5	4	37.3	9.3
EBR15M	9	8	35.1	4.4	EBR15F	10	10	57.6	5.8
EBR16M	5	4	11.2	2.8	EBR16F	10	6	45.4	7.6
EBR17M	9	6	20.2	3.4	EBR17F	11	10	58.9	5.9
EBR18M	9	6	30.3	5.1	EBR18F	6	5	20.2	4.0
EBR19M	5	4	16.8	4.2	EBR19F	8	7	44.4	6.3
EBR20M	7	5	34.6	6.9	EBR20F	10	6	48.0	8.0
EBR21M	9	7	23.4	3.3	EBR21F	5	4	27.7	6.9
EBR22M	7	6	45.0	7.5	EBR22F	7	5	53.7	10.7
EBR23M	2	1	0.0	0.0	EBR23F	2	1	0.0	0.0
–					EBR24F	3	2	22.5	11.3
–					EBR25F	3	1	0.0	0.0
Total	161[a]	122[c]	650.5[e]	–		173	128	944.4	–
Average	7[b]	5[d]	27.3[f]	5.0[g]		7	5	37.3	6.7

Map distances are shown in centimorgans (cM). a; indicates total no. of mapped markers. b; indicates average no. of mapped markers per linkage group. c; indicates total no. of framework makers. d; indicates average no. of framework makers per linkage group. e; indicates total length of linkage groups. f; indicates average length per linkage group. g; indicates average length of intervals between framework markers.

significant orthology relationships with the genes (UGGT2, CPS1, TMED6, MOGAT1, DGKI, CDK17, PDE3B, GANAB and AGXT), which encoded enzymes and have functions in regulating the cell cycles or organism energy metabolism.

4. Discussion

In the male linkage map, 161 markers were assigned to 23 linkage groups, whereas 173 markers were arranged into 25 linkage groups in a female linkage map. Kelp grouper is a diploid fish with 2n = 48 chromosomes (Guo et al., 2003), this number of linkage groups is inconsistent with the haploid chromosome number (n = 24). Thus, more markers are required to merge short length groups in order to obtain the full genome coverage. Considering the marker density of first-generation linkage maps of other species such as Japanese eel (male/female: 6.3/7.2 cM) (Nomura et al., 2011), turbot (sex averaged map, 6.5 cM) (Bouza et al., 2007), grass carp (male/female: 4.2/5.2 cM) (Xia et al., 2010), striped bass (sex averaged map, 5.8 cM) (Liu et al., 2012), tiger pufferfish (male/female: 4.1/7.1 cM) (Kai et al., 2005), barramundi (male/female: 4.7/6.2 cM) (Wang et al., 2007) and Japanese flounder (male/female: 8/6.6 cM) (Coimbra et al., 2003), the average resolution of our map was around in this range, being that male/female is 5.0/6.7 cM between framework markers. Therefore, the marker density of our map would be sufficient for the initial mapping of economically important traits. In addition, a consensus linkage map was constructed from the sex-specific maps. In our study, because of the limited number of co-segregation makers in linkage groups EBR8, EBR11 and EBR23, an integrated map was not constructed. Moreover, because of the absence of the male linkage groups of EBR24 and EBR25, their consensus linkage groups were also excluded. However, an increased linkage group resolution was observed in other linkage groups. Generally, the consensus map can increase the cluster marker density and improve the genome coverage of linkage groups (Zhang et al., 2013). However, considering only one or limited number of reference families were available in this study, sex-specific maps seem to be more precise in QTL detection and genetic breeding programs. Previous research revealed that significant QTL were identified in one sex map due to the differences of recombination as well as the association relationship between genotype and traits (Fuji et al., 2006, 2007). Also, marker order can be checked by comparing sex-specific maps. Hence, in our map, sex-specific maps were used to calculate each parameter of linkage maps.

In the present study, females had a higher recombination rate than males (male:female = 1:1.5) throughout all the linkage groups, although males indicated a higher recombination rate than females in some common marker intervals, which was similar to most fish species. Haldane (1922) and Huxley (1928) suggested that the recombination rate at meiosis becomes different depending on the germline. It is usual that the heterogametic sex has a more suppressed recombination than the homogametic sex. In support of this hypothesis, suppressed recombination rates in the heterogametic sex have been observed in many species. For example, in fish such as grass carp (1:2–2.03, Xia et al., 2010), channel catfish (1:3.18, Waldbieser et al., 2001), rainbow trout (1:1.68–4.31, Danzmann et al., 2005; Rexroad et al., 2008; Sakamoto et al., 2000), European seabass (1:1.48, Chistiakov et al., 2005) and turbot (1:1.3–1.6, Bouza et al., 2007; Ruan et al., 2010). However, with the increasing availability of genomic resources, some exceptions have been found. In medaka (*Oryzias latipes*), pseudo masculinization of genetic females which were hormonally treated produced a lower recombination rate than females to the level shown in

Table 2
Summary of genetic distance of co-segregation markers.

List	LG	Common intervals	Genetic distance (cM)				
			Male[a]	Female[b]	M/F/equivalent[c]	cM for male[d]	cM for female[e]
1	EBR1	Ebr00236FRA/Ebr00284FRA	28.9	26.7	M	45.7	57.3
2		Ebr00284FRA/Ebr00190FRA	11.3	10.2	M		
3		Ebr00190FRA/EawSTR30DB	0	1.1	F		
4		EawSTR30DB/EawSTR8DB	5.5	14.8	F		
5		EawSTR8DB/EfuSTR309DB	0	4.5	F		
6		EfuSTR309DB/Ebr00063FRA	0	0	Equivalent		
7	EBR2	Ebr00056FRA/Ebr00069FRA	0	0	Equivalent	15.6	58.1
8		Ebr00069FRA/EguSTR129DB	1.1	10.1	F		
9		EguSTR129DB/Ebr00257FRA	12.3	40.2	F		
10		Ebr00257FRA/Ebr00185FRA	2.2	7.8	F		
11	EBR3	Ebr00293FRA/Ebr00114FRA	16.1	7.8	M	29.5	49
12		Ebr00114FRA/Ebr00005FRA	2.2	2.3	F		
13		Ebr00005FRA/EfuSTR320DB	6.7	18.6	F		
14		EfuSTR320DB/EawSTR12DB	1.1	2.2	F		
15		EawSTR12DB/Ebr00116FRA	0	4.4	F		
16		Ebr00116FRA/EguSTR122_reDB	3.4	13.7	F		
17	EBR4	EawSTR19DB/EawSTR58DB	0	0	Equivalent	20.2	51.6
18		EawSTR58DB/Ebr00099FRA	0	0	Equivalent		
19		Ebr00099FRA/Ebr00052FRA	0	9	F		
20		Ebr00052FRA/Ebr00200FRA	7.8	17.3	F		
21		Ebr00200FRA/Ebr00047FRA	12.4	25.3	F		
22	EBR5	EawSTR20DB/Ebr00066FRA	4.5	20.4	F	4.5	20.4
23	EBR6	Ebr00041FRA/Ebr00203FRA	0	0	Equivalent	1.1	3.3
24		Ebr00203FRA/PmaSTR301DB	1.1	3.3	F		
25	EBR7	ElaSTR220DB/Ebr00149FRA	5.6	0	M	12.3	2.2
26		Ebr00149FRA/Ebr00218FRA	0	0	Equivalent		
27		Ebr00218FRA/Ebr00158FRA	4.4	2.2	M		
28		Ebr00158FRA/EfuSTR319DB	2.3	0	M		
29	EBR8	Ebr00181FRA/Ebr00204FRA	1.1	0	M	1.1	0
30	EBR9	EquSTR247DB/Ebr00134FRA	0	10.1	F	25.7	46.1
31		Ebr00134FRA/Ebr00199FRA	0	0	Equivalent		
32		Ebr00199FRA/EguSTR148DB	5.6	5.6	Equivalent		
33		EguSTR148DB/Ebr00155FRA	0	0	Equivalent		
34		Ebr00155FRA/EawSTR35DB	7.8	16.8	F		
35		EawSTR35DB/EcoSTR231DB	7.9	12.5	F		
36		EcoSTR231DB/EguSTR157DB	4.4	1.1	M		
37	EBR10	EfuSTR339DB/EawSTR36DB	14.9	32.3	F		40.1
38		EawSTR36DB/Ebr00262FRA	23.7	7.8	M	43.1	
39		Ebr00262FRA/Ebr00265FRA	4.5	0	M		
40	EBR11	EawSTR49DB/Ebr00267FRA	13.4	0	M	13.4	0
41	EBR12	Ebr00106FRA/Ebr00186FRA	0	0	Equivalent	50.6	29.4
42		Ebr00186FRA/Ebr00180FRA	31.3	6.7	M		
43		Ebr00180FRA/Ebr00010FRA	14.9	16	F		
44		Ebr00010FRA/Ebr00179FRA	4.4	6.7	F		
45	EBR13	Ebr00163FRA/Ebr00254FRA	4.5	0	M	21.3	49
46		Ebr00090FRA/EitSTR377DB	10.1	22.9	F		
47		EitSTR377DB/Ebr00263FRA	5.5	2.3	M		
48		Ebr00263FRA/Ebr00292FRA	1.2	23.8	F		
49	EBR14	EfuSTR358DB/Ebr00235FRA	25.9	23.9	M	55.2	37.3
50		Ebr00235FRA/Ebr00209FRA	12.5	5.6	M		
51		Ebr00209FRA/Ebr00187FRA	3.3	7.8	F		
52		Ebr00187FRA/Ebr00024FRA	13.5	0	M		
53	EBR15	Ebr00156FRA/Ebr00064FRA	1.1	13.4	F	35.1	57.6
54		Ebr00064FRA/Ebr00244FRA	0	2.2	F		
55		Ebr00244FRA/Ebr00072FRA	3.3	13.7	F		
56		Ebr00072FRA/Ebr00131FRA	6.7	16.1	F		
57		Ebr00131FRA/Ebr00051FRA	2.3	3.3	F		
58		Ebr00051FRA/Ebr00008FRA	4.4	3.3	M		
59		Ebr00008FRA/Ebr00222FRA	17.3	5.6	M		
60	EBR16	EseSTR78DB/Ebr00138FRA	9	28.5	F	10.1	32.9
61		Ebr00138FRA/EitSTR375DB	1.1	0	M		
62		EitSTR375DB/Ebr00205FRA	0	4.4	F		
63	EBR17	EguSTR150DB/Ebr00153FRA	3.3	15.7	F	20.2	57.8
64		Ebr00153FRA/EguSTR119DB	5.6	6.7	F		
65		EguSTR119DB/EcoSTR261DB	10.2	19.5	F		
66		EcoSTR261DB/Ebr00012FRA	1.1	14.8	F		
67		Ebr00012FRA/Ebr00207FRA	0	1.1	F		
68	EBR18	Ebr00111FRA/Ebr00241FRA	3.3	11.3	F	4.4	19.1
69		Ebr00241FRA/Ebr00091FRA	1.1	4.5	F		
70		Ebr00091FRA/Ebr00202FRA	0	3.3	F		
71		Ebr00202FRA/EitSTR378DB	0	0	Equivalent		
72	EBR19	EacSTR234DB/PlaSTR269DB	1.1	3.3	F	16.8	39.9
73		PlaSTR269DB/Ebr00105FRA	15.7	36.6	F		
74	EBR20	EfuSTR321DB/Ebr00280FRA	21.2	21.2	Equivalent	32.4	43.6

Table 2 (*continued*)

List	LG	Common intervals	Genetic distance (cM)				
			Male[a]	Female[b]	M/F/equivalent[c]	cM for male[d]	cM for female[e]
75		Ebr00280FRA/Ebr00269FRA	2.2	6.7	F		
76		Ebr00269FRA/Ebr00273FRA	0	0	Equivalent		
77		Ebr00273FRA/EguSTR151DB	9	15.7	F		
78		EguSTR151DB/Ebr00043FRA	0	0	Equivalent		
79	EBR21	EguSTR141DB/Ebr00136FRA	4.5	3.3	M	17.9	27.7
80		Ebr00136FRA/Ebr00192FRA	0	0	Equivalent		
81		Ebr00192FRA/Ebr00212FRA	8.9	4.5	M		
82		Ebr00212FRA/Ebr00067FRA	4.5	19.9	F		
83	EBR22	EguSTR147DB/Ebr00152FRA	14.7	14.9	F	16.9	40.2
84		Ebr00152FRA/Ebr00095FRA	2.2	25.3	F		
85	EBR23	Ebr00045FRA/Ebr00249FRA	0	0	Equivalent	0	0
		Total[f]				493.1	762.6
		Recombination ratio[g]				1	1.5

Map distances are shown in centimorgans (cM). a; indicates the genetic distance of co-segregation markers in male linkage group. b; indicates the genetic distance of co-segregation markers in female linkage group. c; indicates which sex exhibits longer genetic distance between co-segregation markers. d; indicates total length of common intervals in each male linkage group. e; indicates total length of common intervals in each female linkage group. f; indicates the total length of common intervals in all 23 linkage groups. g; indicates the average ratio of recombination between sexes.

males (Matsuda et al., 1999). Such observations have yielded new insight that males have lower recombination rates than females, regardless of whether they are the heterogametic sex (Coop and Przeworski, 2007). Kelp grouper has an advantage to study the reason for different recombination rates in males and females, as it is a protogynous hermaphrodite species and an individual undergoes the physiological development of both sexes in its lifecycle as a sex-reversal. The linkage map obtained in this study indicated suppression of recombination in males (second sex). On the other hand, gilthead sea bream is a protandrous hermaphrodite species and an individual has an opposite sex-reversal compared to kelp grouper; nevertheless the linkage map of this fish indicated suppression of recombination in males (first sex, male/female = 1:1.2) (Franch et al., 2006). Considering the results of protogynous and protandrous hermaphrodite species, genetic sex (heterogametic or homogametic sex) is not important to determine recombination rates, and the sexual environment in embryological stage derived from primordial germ cells seems to be the key to produce the different recombination rates between males and females. Further studies on the construction of the linkage maps using before and after sex-reversal of the same individual would enable insights on this subject.

According to our knowledge, this map is the first linkage map in the subfamily Epinephelinae. Microsatellite markers genetically present highly conserved flanking regions in a diverse number of closely related species (Scribner et al., 1996). Using microsatellite markers developed from related species on fish research has led to extend the application to construct genetic linkage maps. Markers isolated from Atlantic halibut were approximately 9% and 5% available for mapping barfin flounder and spotted halibut, respectively (Ma et al., 2011) and markers from seven kinds of flatfish species showed 38% availability for mapping Atlantic halibut (Reid et al., 2007). Our findings also supported this result. Markers obtained from Epinephelinae species (*Epinephelus acanthistius*, *Epinephelus akaara*, *Epinephelus awoara*, *Epinephelus coioides*, *Epinephelus fuscoguttatus*, *Epinephelus guttatus*, *Epinephelus itajara*, *Epinephelus lanceolatus*, *Epinephelus quernus*, *Epinephelus septemfasciatus*, *Cephalopholis fulva*, *Mycteroperca microlepis*, *Plectropomus laevis*, *Paretroplus maculatus*) worked well in the kelp grouper analysis. In total, 21% of 360 loci isolated from other groupers in this study could be used to construct kelp grouper genetic linkage maps, which were shown to be effective for common usage in groupers. Lack of specific genetic linkage maps for grouper species has delayed their genetic improvement for aquaculture. However, as grouper microsatellites can be widely amplified among closely related species, using "common" markers among related species will accelerate the construction of linkage maps in other groupers and allow the clarification of the inter-relationships among groupers.

Table 3
Summary of male and female genetic linkage maps of kelp grouper.

	Male genetic linkage map	Female genetic linkage map
No. of genetic linkage groups	23	25
Minimum length of genetic linkage group (cM)	0	0
Maximum length of genetic linkage group (cM)	58.5	62.9
Total length (cM)		
G_{total}	650.5	944.4
Estimated genome length (cM)		
G_{e1}	952.7	1402.8
G_{e2}	967.1	1406.3
G_e	959.9	1404.6
Genome coverage %		
C_f	67.80%	67.20%

Map distances are shown in centimorgans (cM). The estimated genome length (G_e) is estimated using 2 different methods: G_{e1} and G_{e2}. Total length of all linkage groups is named as G_{total}. The genetic linkage map coverage of C_f is denoted by G_{total}/G_e.

Blast sequences of marker located in the genetic linkage map may not only facilitate understanding fish genome origination and evolution by comparative genome analysis (Danzmann et al., 2008; Kasahara et al., 2007), but also benefit to identify potential genes in the candidate QTL regions (Wang et al., 2011a,b). In addition, if the target fish exhibits an orthology relationship with related species of which whole genome sequences have been assembled, this will enable the prediction of candidate genes more comprehensively. In our study, although some alignments did not exhibit significant similarity, we identified several potential candidate genes involved in physiological and biochemical reactions (i.e., ELTD1 and ROBO3). ELTD1 may function in both leukocyte adhesion and migration in inflamed epidermal and mucosal tissue (Leemans et al., 2004) and this gene plays a role in surface immune defense (Harkensee et al., 2013). However, its function in fish has not been studied yet. Gonadotropin-releasing hormone (GnRH) neuronal system up-regulates reproduction in mammals. ROBO3 plays a major role in the development of the GnRH neuronal system and is relevant to the reproduction (Cariboni et al., 2012). Markers in LG 1 of kelp grouper exhibited the largest number of syntenic candidate genes, which infers that LG1 may be important for further QTL analysis. Here, our blast result could be the first step that serves as a reference for further identifying candidate genes in the regions where significant QTL have been mapped. Future gene clones and their functional expression are required, in order to understand the molecular basis of genes in the possible QTL regions underlying important traits in kelp grouper. Moreover, previous research revealed that markers derived from cross species could be used for QTL analysis (Reid et al., 2005; Somorjai et al., 2003) and

comparative mapping (Rexroad et al., 2008). Hence, in our study, it is a possibility that both of the markers derived from kelp grouper genome and cross species genomes could be suitable as an anchor for the potential candidate genes for further QTL detection.

Research on kelp grouper aquaculture has continued for more than 40 years in Japan. Although advances in management have been successfully applied to kelp grouper aquaculture, the yield is still at a low level for markets due to some problems mentioned before. Genetic improvement would be an effective approach to solve these problems, compared with traditional tools, which have been found to be effective for flounder and salmonid aquaculture. Based on genetic linkage map, QTL or even gene(s) underlying economically important traits, such as growth, disease resistance and survival might be detected. Furthermore it will assist the best selection of "broodstock" to construct high quality strains for further genetic improvement of this species. In addition, considering groupers might share a large number of orthologous genome, our map might provide basic information towards comparative analysis conserved QTL affecting economically important traits among groupers. Also, genetic linkage map might be possible to apply in comparative genome analysis of grouper genome rearrangement among different species in order to give an insight into grouper genome.

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