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Constructing a genetic linkage map and mapping quantitative trait loci for skeletal traits in Japanese flounder

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Abstract: A genetic linkage map of Japanese flounder was constructed using 165 doubled haploids (DHs) derived from a single female. A total of 574 genomic microsatellites (type II SSRs) and expressed sequence tag (EST)-derived markers (EST-SSRs) were mapped to 24 linkage groups. The length of linkage map was estimated as 1270.9 centiMorgans (cM), with an average distance between markers of 2.2 cM. The EST-SSRs were used together with type II SSR markers to construct the Japanese flounder genetic linkage map which will facilitate identify quantitative trait locus (QTL) controlling important economic traits in Japanese flounder. Thus, twelve skeletal traits at 2 years of age were measured for all DHs. Forty-one QTLs were detected on 14 linkage groups and totally account for a small proportion of phenotypic variation (4.5 to 17.3%). Most of QTLs detected distribute on linkage groups 5 (9 QTLs), 8 (9 QTLs), 9 (5 QTLs) and 20 (4 QTLs), in which, some QTLs perform the pleiotropy.

Key words: doubled haploid; linkage map; type II SSR; EST-SSR; Japanese flounder; skeletal traits

Introduction

Genetic linkage maps have been developed for many commercial fish species, such as tilapia (Kocher et al. 1998), catfish (Liu & Dunham 1998), common carp (Sun & Liang 2004) and half-smooth tongue sole (Liao et al. 2009), which can be used to locate chromosomal regions where DNA markers co-segregate with quantitative traits (quantitative trait loci, QTL). In most of the studies, backcross or F2 progeny have been used as mapping population, but, they are only useful until the DNA supply from an individual is exhausted. In contrast, homozygous doubled haploid (DH) lines constitute a resource that can be used for mapping and research for as long as the lines are perpetuated (Young et al. 1998). In future studies, markers can be continually added to the map and the DNA or lines can also be sent to other labs for additional marker and trait analysis (Burr et al. 1988). But for now, linkage map based on DH progeny in cultured fish species has only been constructed in rainbow trout (Young et al. 1998; Nichols et al. 2003). This was partly due to DHs often performs a high rate of mortality and individuals can seldom survive to stages of sexual maturity.

Japanese flounder, *Paralichthys olivaceus* (Temminck et Schlegel, 1846), is one of the aquaculture fish species for seawater cultivation in China, Japan and Korea. In order to improve the traits of interest, a coordinated breeding program that includes phenotypic selection, family selection, and marker-assisted selection (MAS) based on QTL needs to be carried out. Thus, an important step is to develop high-density genetic map in such a genetic improvement process. Genetic linkage maps based on molecular markers at a large number of sites in the genome constitute an essential prerequisite to identify individual loci controlling targeted traits. In previous studies, the published Japanese flounder maps (Combria et al. 2003; Kang et al. 2008; Castaño-Sánchez et al. 2010) have provided the framework for identifying a single major controlling the resistance to lymphocystis disease in hybrid family (Fuji et al. 2006). A new linkage map of Japanese flounder constructed by Song et al. (2012) and QTL associated with four growth traits were mapped. The studies on mapping QTL for skeletal traits are very important in exploring questions about the evolution of the system as a whole in vertebrates as well as in biomedical research on the genetics of bone growth (Kenney-Hunt et al. 2008). So far, many published articles on the ge-

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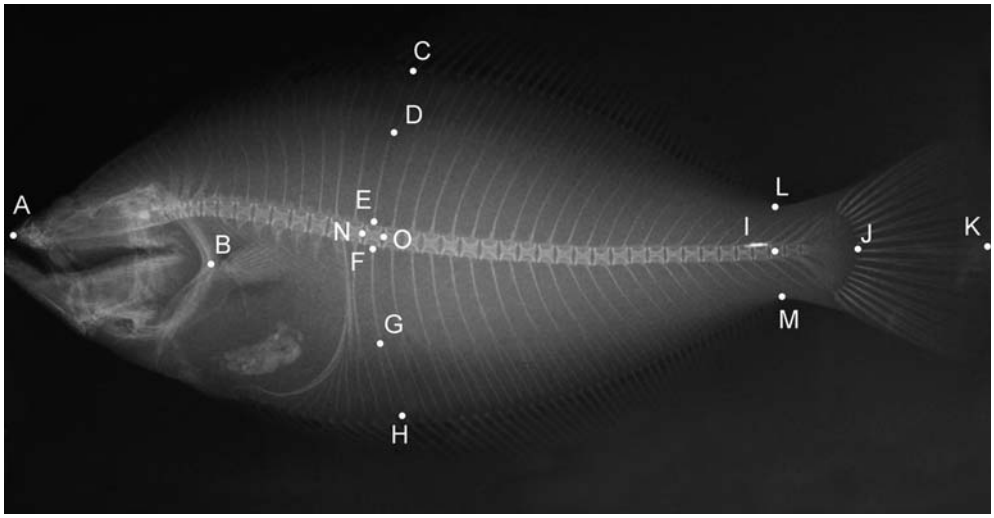


Fig. 1. The measurement standards of skeletal traits. AK: Total length; AJ: Body length; CH: Body depth; AB: Head length; IJ: Caudal peduncle length; LM: Caudal peduncle width; NO: Vertebrae length; EF: Vertebrae Width; DE: Neural spine length; FG: Haemal spine length; CD: Interneural spine length; GH: Interhaemal spine length. The measurement unit is centimeter.

netic architecture of skeletal traits using QTL analysis mainly reported in the mammal (Cheverud et al. 2001; David et al. 2005) while rarely in fish species.

In this article, we reported a Japanese flounder map constructed by 481 type II SSR and 141 EST-SSR markers and used a DH population. Among the mapped markers, 164 are new to this map including EST-SSRs which have advantages of representing coding regions of the genome, and direct associations can be made between genotype and phenotype leading to identification of QTL underlying the traits of interest (Rudd 2003). Therefore, using this map, we attempt to identify QTLs of major skeletal traits, which will provide a valuable resource for establishing a marker-assisted breeding scheme.

Material and methods

Mapping population

Doubled haploids in Japanese flounder were produced from one female following a reported standard protocol of mitotic gynogenesis (Yamamoto 1999). In gynogenesis, sperm does not contribute genetic material to the offspring, but acts as a stimulator for embryogenesis. To ensure that any surviving offspring are real gynogenetic, the use of suitable heterologous sperm is a promising strategy. Sperm of red sea bream is the best choice in this study because they can initiate the development of flounder eggs, but no hybrid individuals will survive.

Flounder eggs were fertilized with ultraviolet-irradiated red sea bream sperm at the dose of 40–50 mJ cm^{-2}. The cleavage of eggs was inhibited with hydrostatic pressure (650 kg cm^{-2}) for 6 min after 60 min fertilization. Subsequently, eggs were transferred to 17 ℃ seawater for incubation. These DHs and their dam were used to construct the genetic linkage map. Genomic DNA was extracted from a fragment of fin clip from parental fish and progeny using a routine method (Blin & Stafford 1976).

Trait measurements

A total of 165 DHs were measured at 2 years of age. Prior to measurements, each individual was anaesthetized with 2-phenoxyethanol to avoid handling stress. At measurements, a photo of each fish skeleton was taken with portable and high frequency medical diagnosis X-ray machine (LX-20A, Beijing Longsafe Imaging Technology Co., Ltd, China) at a standard height perpendicular to the fish. All photos were provided with a reference scale length. In addition to this, total length, body length, body depth, head length, caudal peduncle length, caudal peduncle depth, vertebrae length, vertebrae width, neural spine length, haemal spine length, interneural spine length and interhaemal spine length were also recorded. The positions on which the body measurements were taken are shown in Fig. 1.

Microsatellite marker

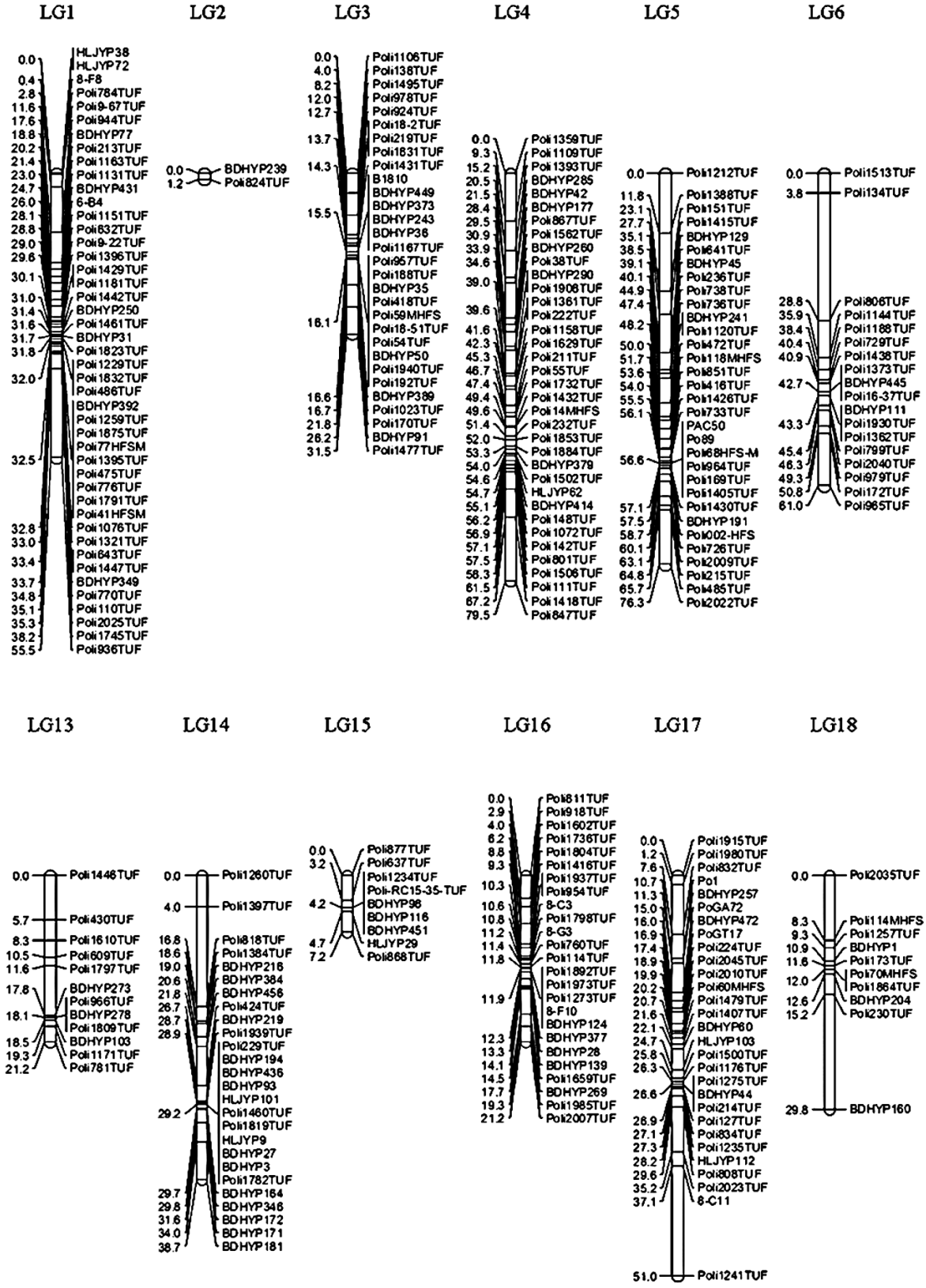
A total of 1007 type II SSR markers used in this study were taken from GenBank/EMBL/DDBJ database. In addition, 15268 EST sequences retrieved from the GenBank/EMBL/DDBJ databases were screened for mono-, di-, tri- and tetra-nucleotide SSRs using Tendem repeat finder (Benson 1999). Primers were designed with PRIMER3 software (Rozen & Skaletsky 2000). Four hundred and eighty-four EST-SSR primer pairs were developed and given name begun with "BDHYP". The polymorphisms and mapping feasibility of these markers were evaluated by genotyping of parental DNA.

Microsatellite genotyping

PCR was performed in a reaction mixture (15 μl) containing: 30–50 ng of genomic DNA, 2 pmol of each primer, 1 U of Taq polymerase (Takara), and 1× PCR buffer (50 mM of KCl, 10 mM of Tris-HCl, 1.5 mM of MgCl2, pH 8.3). Thermal cycles were carried out as follows: initial denaturing step (3 min at 94 ℃); 28 cycles (94 ℃ for 30 s, 30 s at 72 ℃, 72 ℃ for 30 s) and a final extension step (10 min at 72 ℃). PCR products were electrophoresed on 8% (wt/vol) denatured polyacrylamide gel (19:1 acrylamide: bis-acrylamide and 7 M urea) and were identified by silver nitrate staining (Liao et al. 2007).

Map construction

Linkage analyses were conducted using Joinmap 4.0 software (Van Ooijen et al. 2006), which can incorporate a wide range of polymorphism types. The linkage groups were established with a LOD=4.0. Map distances in centimorgans were calculated using Kosambi's mapping function. Linkage groups were numbered according to previous map (Castaño-Sánchez et al. 2010). The graphic representation of the linkage groups was created by MapChart 2.1 (Voorrips 2002).



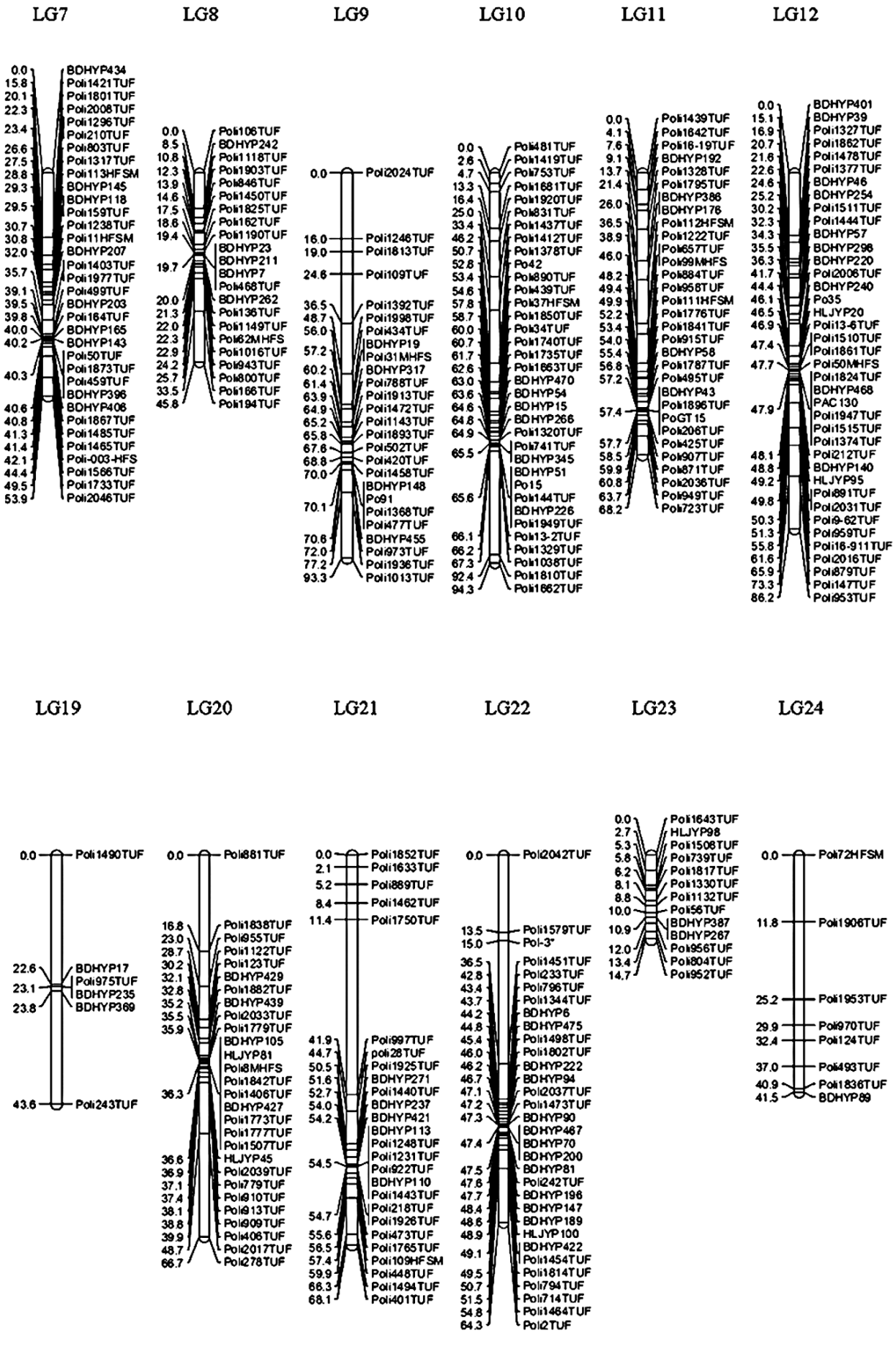


Fig. 2. Linkage map for Japanese flounder. The marker distances are indicated in Kosombi centiMorgans.

QTL analysis
MapQTL (version 4.0) (Van Ooijen et al. 2002) was used to identify QTL following a method of MQM (Jansen 1994; Jansen & Stam 1994) which included two steps. Firstly, putative QTLs were found by the approach of interval mapping according to an LOD $\geq$ 2.0. Secondly, markers close to putative QTLs were selected as co-factors, then a multiple-QTL model were performed. QTLs were considered at the point of maximum LOD score in each linkage group. The rule for naming QTLs was: trait abbreviation-linkage group-number of QTLs.

Results

Microsatellite analysis
Out of a total of 1007 type II SSR markers tested, 481 (47.8%) were heterozygous in the female parent of mitotic gynogens. An additional 165 doubled haploids were genotyped for these 481 type II SSR markers. Four hundred and eighty-four microsatellite primer pairs were designed from EST sequences, of which, 141 (29.1%) were proved to be polymorphic in our mapping population. In total, 481 type II SSR and 141 EST-SSR markers were used for the linkage analysis.

Map construction
The genetic linkage map was constructed using 622 markers (481 type II SSRs and 141 EST-SSRs) with 165 double haploid offspring. A total of 574 markers (446 type II SSRs and 128 EST-SSRs) were assigned to the framework map, which consisted of 24 LGs (linkage groups) spanning 1214.7 cM (Fig. 2). Forty-eight markers (35 type II SSRs and 13 EST-SSRs) were not linked to any LG. The average number of markers for the 24 groups was approximately 23.9 (18.6 type II SSRs and 5.3 EST-SSRs per group). The length of each LG ranged from 1.2 to 94.3 cM with an average of 2.12 cM, and the average marker spacing of each group varied from 0.88 to 8.72 cM with an average of 2.2 cM for the whole map. This map displays 458 unique positions.

The chi-square test was used to test segregation distortion pattern, where the level of significance was considered as 5%. A total of 86 markers (55 type II SSRs and 31 EST-SSRs) on the linkage map showed significant segregation distortion. The major significantly distorted markers appeared on the LG1, LG3 and LG7. The distorted markers were retained because they did not influence the order of other markers on the linkage map.

A total of 400 previously-mapped type II markers (Castaño-Sánchez et al. 2010) were also found in our linkage map. The linkages and order of markers in this map were largely concordant with those in the second generation linkage map. Only eleven of the markers mapped to the different linkage group (Table 1).

Mapping QTL for skeletal traits
A total of 12 traits are observed and 622 markers covering a genome of length 1214.7 cM are genotyped for the DH line. Table 2 tabulates parameter estimates of QTLs detected with MapQTL software. Most of QTLs detected distribute on linkage groups 5 (9 QTLs), 8 (9 QTLs), 9 (5 QTLs) and 20 (4 QTLs), in which, some QTLs showed pleiotropy. For instance, the QTL on marker 'Poli1825TUF' simultaneously governing body depth, vertebrae length, neural spine length, haemal spine length and interhaemal spine length. Especially, many QTLs are identified on makers: 2 markers for head length, 5 markers for caudal peduncle length, 3 markers for caudal peduncle depth, 3 markers for vertebrae height and 3 markers for interhaemal spine length. The proportions of phenotypic variation explained by the detected QTLs ranged from 4% to 17%. The largest heritability is 0.17 of the QTL for head length on linkage group 19 and neural spine length on linkage group 8, followed by 0.16 of the QTL for body depth on linkage group 8 and the smallest one is 0.04 of the QTL for vertebrae length on linkage group 8.

Table 1. Different marker location between the current and the previous (the second generation) linkage maps of Japanese flounder.

Number	Marker	Current LG	Previous LG
1	Poli1229TUF	LG1	LG17
2	Poli472TUF	LG5	LG21
3	Poli641TUF	LG5	LG2
4	Poli729TUF	LG6	LG11
5	Poli481TUF	LG10	LG17
6	Poli1810TUF	LG10	LG3
7	Poli99MHFS	LG11	LG2
8	Poli206TUF	LG11	LG8
9	Poli406TUF	LG20	LG13
10	Poli909TUF	LG20	LG24
11	Poli473TUF	LG21	LG5

Discussion

We constructed a new genetic linkage map for the Japanese flounder from mapping efforts in DH line. Generally, backcross (BC) population is the simplest type of mapping population. They shared similar advantage with DH: only having two possible genotypes at one locus and easily to be analyzed during QTL mapping (Mutshinda & Sillanpää 2010). However, BC population could be a temporary population for their heterozygous (Semagn et al. 2006). The DH population developed here represented an extremely valuable resource (Burr et al. 1988). Markers could be continually added to this map in the future studies and the DH line or their DNA can also be sent to other labs for additional analysis.

Compared to the former ones, this genetic linkage map was constructed with 574 markers including 446 type II SSRs and 128 EST-SSRs, which makes it denser than the maps published before the year of 2008 (Coimbra et al. 2003; Castaño-Sánchez et al. 2008; Kang et al. 2008), but not presently the densest flatfish linkage map. The second generation genetic linkage map of the Japanese flounder were constructed by 1375 markers (Castaño-Sánchez et al. 2010). However, the female

Table 2. QTLs of skeletal traits detected in DH population of Japanese flounder.

Trait	QTL	LOD	Linkage group	Position/cM	Marker interval	Additive effect	% expl*
Total length	TL-5-1	2.33	5	59.7	Poli-002-HFS∼Poli726TUF	–4.65	10.4
	TL-16-1	2.88	16	17.7	BDHYP269	–2.01	11.7
Body length	BL-5-1	2.74	5	58.7	Poli-002-HFS	–4.61	12.4
	BL-16-1	2.48	16	17.7	BDHYP269	–1.76	10.7
	BL-22-1	2.37	22	8.0	Poli2024TUF∼Poli1579TUF	–1.51	11.4
Body Depth	BD-8-1	2.45	8	18.6	Poli1825TUF∼Poli162TUF	1.00	16.3
	BD-8-2	2.30	8	23.9	Poli1016TUF∼Poli943TUF	–1.76	13.7
Head length	HL-5-1	2.75	5	54.0	Poli416TUF	0.35	7.7
	HL-19-1	2.71	19	31.8	BDHYP369∼Poli243TUF	0.74	17.1
	HL-20-1	2.23	20	50.7	Poli2017TUF∼Poli278TUF	–0.31	6.0
	HL-21-1	2.11	21	54.0	BDHYP237	–0.27	4.5
Caudal peduncle length	CPL-5-1	2.82	5	51.7	Poli118MHFS	–0.42	7.3
	CPL-5-2	2.59	5	56.1	Poli733TUF	0.17	7.2
	CPL-8-1	2.45	8	8.5	BDHYP242	0.15	6.4
	CPL-9-1	2.54	9	63.9	Poli1913TUF	–0.17	6.8
	CPL-22-1	2.74	22	64.3	Poli2TUF	–0.18	7.0
Caudal peduncle depth	CPD-5-1	2.44	5	56.1	Poli733TUF	0.21	10.8
	CPD-8-1	2.32	6	39.5	Poli166TUF∼Poli194TUF	0.23	13.4
	CPD-15-1	2.11	15	7.2	Poli868TUF	0.20	9.0
	CPD-21-1	2.08	21	0.0	Poli1852TUF	0.21	7.8
Vertebrae length	VL-5-1	2.15	18	8.0	Poli2035TUF∼Poli114MHFS	0.01	8.7
	VL-8-1	2.02	8	18.6	Poli162TUF	0.02	5.4
	VL-9-1	2.07	9	39.5	Poli1932TUF∼Poli1998TUF	0.01	6.3
	VL-17-1	2.06	17	35.2	Poli2023TUF	–0.03	8.7
Vertebrae width	VW-5-1	2.57	5	57.5	BDHYP191	0.05	14.1
	VW-8-1	2.16	8	0.0	Poli106TUF	0.05	12.8
	VW-9-1	2.06	9	93.2	Poli1936TUF∼Poli1013TUF	–0.05	11.7
	VW-20-1	2.67	20	39.9	Poli406TUF	–0.05	13.7
Neural spine length	NSL-8-1	2.55	8	18.6	Poli1825TUF∼Poli162TUF	0.28	17.3
	NSL-8-2	2.02	8	23.9	Poli1016TUF∼Poli943TUF	–0.48	12.7
Haemal spine length	HSL-8-1	2.11	8	18.6	Poli1825TUF∼Poli162TUF	0.34	15.3
	HSL-20-1	2.17	20	32.1	Poli123TUF∼BDHYP429	–0.28	12.2
	HSL-20-2	2.11	20	38.8	Poli909TUF	–0.27	11.8
Interneural spine length	INSL-5-1	2.43	5	51.7	Poli118MHFS	–0.31	10.9
	INSL-5-2	2.11	5	60.1	Poli002-HFS∼Poli726TUF	–0.33	9.6
	INSL-9-1	2.07	9	61.4	BDHYP317∼Poli788TUF	–0.23	9.8
Interhaemal spine length	IHSL-1-1	2.02	1	38.2	Poli1745TUF	0.12	6.0
	IHSL-8-1	3.14	8	18.6	Poli1825TUF∼Poli162TUF	0.21	12.4
	IHSL-9-1	2.35	9	78.2	Poli1936TUF∼Poli1013TUF	0.17	7.5
	IHSL-13-1	2.04	13	18.1	Poli966TUF	0.17	7.8
	IHSL-22-1	2.13	22	64.3	Poli2TUF	0.11	5.9

*%expl: the percentage of the phenotypic variation explained by the QTL.

maps have only 184 unique positions. In this research, the order of markers was basically consistent with that in the second generation map, furthermore, these markers were more evenly distributed on the LGs. In this study, the linkage map has 458 unique positions which may show that DH population has a higher mapping efficiency.

Actually, 1007 type II SSR and 484 EST-SSR markers were screened for female parent in this research. The number of homozygous markers attained to 869, which preventing their assignment on the map. The percentage of informative polymorphic EST-SSR (29.1%) markers was much lower than that of type II SSR markers, which showed that ESTs from expressed regions were more conserved than non-coding regions of the genome (Dreisigacker et al. 2004; Varshney et al. 2005). While, ESTs represented coding regions of the genome, direct associations could be made between genotype and phenotype leading to identification of quantitative trait loci (QTL) underlying the traits of interest because ESTs were products from transcript abundances (Rudd 2003).

The growth trait of fish has long been studied, due to its importance for aquaculture. However, most re-

search mainly focused on body weight or morphological traits. In our study, we firstly reported the markers linked to skeletal traits in Japanese flounder. The main difficulty for skeletal traits research in fish was hard to obtain the phenotypic value. To resolve this difficulty, we used an X-ray equipment to scan each fish and measured the skeletal traits on photos. After mapping experiment, a total of 41 QTLs were identified and mainly distributed on LG5, LG8, LG9, LG20 and LG22. Many QTLs clustered in adjacent position of chromosome, which showing they might be controlled by the same genomic regions. For instance, the QTLs of L3, L7, L9, L10 and L12 were identified on the same position in LG8, which indicating the effect of gene pleiotropy (Quarrie et al. 2005). In addition, some QTLs located in identical genomic regions, such as QTLs of L3 and L9, which demonstrated these traits could be controlled by the same gene (Doganlar et al. 2002). Compared to Song et al. (2012), QTL for body depth was mapped onto LG14 which was on LG8 in our study. This may mainly due to the differences of mapping population, and may also be associated with the differences of the marker number in linkage map and measurement of traits. The growth data obtained from skeletal measurement were usually more stable and reproducible.

The current results will provide useful information for establishing breeding program by molecular marker-assisted selection in Japanese flounder. We are currently undergoing further research and development in denser genetic linkage map with more types of marker in order to identify more major QTLs associated with growth traits or resistance to disease.

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