Current Zoology 59 (1): 99–108, 2013

Construction of a microsatellite-based genetic linkage map for half-smooth tongue sole *Cynoglossus semilaevis*

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Abstract The half-smooth tongue sole *Cynoglossus semilaevis* is an important cultured marine fish and a promising model fish for the study of sex determination. Sex-specific genetic linkage maps of half-smooth tongue sole were developed with 567 markers (565 microsatellite markers and two SCAR markers). The parents and F1 progeny (92 individuals) were used as segregating populations. The female map was composed of 480 markers in 21 linkage groups, covering a total of 1388.1 cM, with an average interval 3.06 cM between markers. The male map consisted of 417 markers in 21 linkage groups, spanning 1480.9 cM, with an average interval of 3.75 cM. The female and male maps had 474 and 416 unique positions, respectively. The genome length of half-smooth tongue sole was estimated to be 1522.9 cM for females and 1649.1cM for males. Based on estimations of map length, the female and male maps covered 91.1% and 89.8% of the genome, respectively. Furthermore, two female-specific SCAR markers, f-382 and f-783, were mapped on LG15f (linkage group 15 in female maps). The present study presents a mid-density genetic linkage map for half-smooth tongue sole. These improved genetic linkage maps may facilitate systematic genome searches to identify quantitative trait loci (QTL), such as disease resistance, growth and sex-related traits, and are very useful for marker-assisted selection breeding programs for economically important traits in half-smooth tongue sole [*Current Zoology* 59 (1): 99–108, 2013].

Keywords Half-smooth tongue sole, *Cynoglossus semilaevis*, Microsatellite, Genetic linkage map, MAS

Genetic linkage maps have become important tools in many areas of genetic research. To perform a linkage study, it is necessary to genotype and map a large number of the available genetic markers on mapping families. Microsatellites comprise an excellent choice for genomic mapping due to their abundance in most vertebrate genomes, genomic distribution, high polymorphism and ease of typing via PCR. Meanwhile, simple sequence repeat (SSR) alleles are typically co-dominant, and their polymorphisms can be scored in either a simple polyacrylamide gel separation format or with high-throughput capillary arrays. Genetic linkage maps based on microsatellite markers have been generated for economically important marine fishes such as salmon (Gilbey et al., 2004), tilapia (Lee et al., 2005), European sea bass (Chistiakov et al., 2005), rainbow trout (Guyomard et al., 2006), sea bream (Senger et al., 2006), barramundi (Wang et al., 2007), catfish (Wang et al., 2007), grass carp (Xia et al., 2010), Japanese flounder (Castaño-Sánchez et al., 2010) and Asian sea bass (Wang et al., 2011).

The half-smooth tongue sole *Cynoglossus semilaevis* is a commercially valuable flatfish widely distributed in Chinese coastal waters. Due to its commercial value, easy domestication and natural resource depletion, half-smooth tongue sole has been selected as a promising species for aquaculture. It is one of the most popular marine species in Chinese aquaculture. In addition, half-smooth tongue sole females grow two to three times larger and faster than males. Based on G-banding patterns analysis, it was confirmed that, in addition to having 20 euchromosome pairs, there is a pair of sex chromosomes (chromosome Z and W), and sex in this species is determined by a WZ/ZZ chromosomal system (Zhuang et al., 2006). These characteristics suggest that half-smooth tongue sole has great potential for the production of all-female stock, as well as for studying the mechanisms of both genome evolution and sex determination.

Half-smooth tongue sole breeding is still in its infancy. Like other aquaculture species, the production of half-smooth tongue sole is often affected by outbreaks

Received Apr. 1, 2012; accepted June 27, 2012.

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of deadly infectious diseases caused by bacteria, viruses or protozoan pathogens. The traditional methods of genetic improvement of quantitative traits have relied mainly on phenotype (Falconer and Mackay, 1996), which are easily influenced by environmental factors. It is generally accepted that marker-assisted selection (MAS) accelerates genetic improvement in a relatively short period, especially when the target characteristics are disease-related and there is a sufficient amount of observed genetic variation in a given trait. Therefore, a genetic map constructed from a population segregated for a trait of interest is required for QTL identification. Information on genetic markers associated with QTL can be used in MAS breeding programs to identify and select individuals carrying desired traits. QTL for growth, disease resistance and stress response have been mapped in only a few species, such as rainbow trout (Ozaki et al., 2001), tilapia (Cnaani et al., 2003), salmon (Reid et al., 2004), Japanese flounder (Fuji et al., 2006), guppy (Tripathi et al., 2009) and European seabass (Massault et al., 2010).

Recently, a number of genetic studies in half-smooth tongue sole have been reported, including the development of microsatellite markers (Liao et al., 2009; Miao et al., 2011; Sha et al., 2011;) and female-specific DNA markers (Chen et al., 2007; Ma et al., 2009), construction of BAC libraries (Shao et al., 2010), molecular marker-assisted sex control (Chen et al., 2008), the characterization of certain sex-related genes (Deng et al., 2009) and artificial gynogenesis (Chen et al., 2009). A low-density genetic linkage map was constructed for half-smooth tongue sole (Liao et al., 2009); however, this map has provided very little information on the genomic organization of this important marine species. The half-smooth tongue sole breeding community lacks a detailed genetic linkage map to facilitate the breeding process. In the present study, we constructed a mid-density microsatellite genetic linkage map in half-smooth tongue sole, and identified sex-linked SCAR markers on linkage maps.

1 Materials and Methods

1.1 Mapping family

A full-sib family of half-smooth tongue sole was constructed and used for the development of a genetic linkage map. The male parent was selected from a group of fish derived from a wild population. The female parent was selected from a cultured population. Experimental crossing was conducted at the MingBo Aquaculture Company (Yantai, China). Induction of the maturation of broodstock and artificial fertilization of sperm and eggs were carried out as described previously (Chen et al., 2009). Ninety-two F_1 offspring from the mapping family were collected, including forty-six females and forty-six males, and stored in absolute ethanol until DNA extraction. Genomic DNA of the two parents and progeny was extracted following phenol/chloroform procedures with RNase treatment (Sambrook and Russell, 2001).

1.2 Microsatellite markers

A total of 2,276 half-smooth tongue sole microsatellite markers were tested for segregation across a set of eight progeny individuals. These microsatellite markers were recruited from three sources: (1) The first set of 1200 microsatellite markers was developed from genome sequencing. (2) The second set of 965 microsatellite markers was developed through the construction of microsatellite enriched libraries and EST libraries (Liao et al., 2009; Miao et al., 2011; Sha et al., 2011). (3) The remaining 111 markers were developed from public databases and the literature (Liu et al., 2007; Liu et al., 2008; Wang et al., 2008; Zhong et al., 2009).

1.3 SCAR markers

Two female-specific SCAR (sequence-characterized amplified region) markers were used from half-smooth tongue sole in the mapping family (Marker name: F-382, Forward primer: ATTCACTGACCCCTGAGAGC, Reverse primer: AACAACTCACACACGACAAATG; F-783, Forward primer: TGTTCTTGTCTTCGCTCCCT, Reverse primer: AGGTGTAACCATCAACTTTTTC). Detailed PCR amplification procedures are described by Chen et al. (2007) and Ma et al. (2009).

1.4 Genotyping

Primers flanking the microsatellite regions were designed using Primer 3. All primers were designed for a 57.5°C annealing temperature, a total amplification product size of 150–300 bp. All microsatellite markers were used to genotype eight progeny for screening the segregation markers in the mapping population. The microsatellite markers that produced polymorphic fragments were used in subsequent genotyping of the parents and 92 progeny to construct linkage maps. Amplification reactions were carried out in a 15μl volume consisting of 10×Taq buffer, 0.5 U Taq polymerase, 0.6mM dNTP (+$MgCl_2$), 0.6 μM of each primer and 10-30ng template DNA. The final volume was adjusted with sterile distilled water. Amplifications were performed in an ABI Veriti 96 well thermal cycler. The PCR amplifications were performed under the following conditions: 95°C for 5 min, followed by 32 cycles at

95°C for 30 s, 57.5 for 30 s and 72°C for 30 s, and the final extension was 72°C for 10 min. The PCR products were separated on 8% polyacrylamide gels (PAGE) and visualized by silver staining (Bassam et al., 1991).

1.5 Linkage analysis

Genetic marker data were scored according to the definition of JoinMap 4.0 (Van Ooijen, 2006). Linkage groups were constructed independently for males and females. Linkage groups with genetic markers on individual maps were merged to create an integrated map using the "Join-combine groups for map integration" command. All statistical analyses described below were made using the same software using a cross-pollinating (CP) type population, which handles F1 outbreeding population data containing various genotype configurations. Pairwise analyses were performed and markers were sorted in linkage groups at a minimum LOD score of 4.0. The "locus genotype frequency" function calculated the chi-square values for each marker to test for the expected Mendelian segregation ratio. The linkage distances were estimated for each LG assuming the Kosambi mapping function. All weak linkage markers were excluded to ensure a correct marker order. Although distorted segregation markers normally are excluded from linkage analysis, the use of the independent LOD score, one of the grouping parameters provided by JoinMap4.0, allows these markers to be included (You et al., 2010). This test for independence is not affected by segregation distortion and leads to a less spurious linkage.

1.6 Genome size and coverage

The estimated genome length (Ge) of the consensus female and male genome was estimated using two different methods. First, Genome Estimation size 1 (Ge1) was calculated by adding 2s to the length of each genetic linkage group to account for the chromosome ends, where s was the average spacing of the genetic linkage map. The first method estimates s on a genome scale (Fishman et al., 2001). Genome Estimation Size 2 (Ge2) was calculated by multiplying the length of each genetic linkage group by (m+1)/(m−1), where m was the number of loci in each genetic linkage group. The second method estimates the average spacing for each chromosome independently (Chakravarti et al., 1991). The estimated genome size (Ge) for each sex was taken as the average of the two estimates. Observed genome length was taken as the total length (Goa) considering all linkage groups, triplets and doublets (Cervera et al., 2001). The map coverage, Coa, was calculated as Goa/Ge (Liao et al., 2007).

2 Results

2.1 Genetic markers and segregation distortion

To obtain useful microsatellite markers for linkage analysis, we examined the segregation patterns of 2,276 markers in the mapping family. Amongst the 2,276 markers, 718 microsatellite markers (31.5%) were polymorphic in either the male or female of the family. Segregation distortion from that expected under Mendelian inheritance was found in 165 (23.0%) of 718 microsatellite markers.

2.2 Sex-linked markers

Sex-specific molecular markers are a useful genetic resource for studying sex- determination mechanisms and controlling fish sex. We mapped two sex-linked SCAR markers (f-382 and f-783) to the female map LG15f (Fig. 1). The presence of sex-linked markers suggested the possibility of female heterogamety (ZZ male; WZ female) in half-smooth tongue sole, which is confirmed by the presence of a large heteromorphic sex chromosome in the females of this species. Therefore, LG15 should correspond to the sex chromosome.

2.3 Linkage Analysis

A total of 720 demonstrably heterozygous markers were available for mapping. Among these 720 loci, 613 markers were used to construct the female map, and 567 markers were used to construct the male map. Thirty-two microsatellite markers did not exhibit any significant linkage to any other markers. When the total of 718 effective microsatellite markers and two SCAR markers were analyzed, 567 markers were found to be located on the linkage map containing 21 linkage groups (LGs) at a LOD threshold value of 4.0.

2.4 Sex-specific maps

Significant linkages were identified for 720 genetic markers, including a total of 718 microsatellite loci and two SCAR markers. However, 154 microsatellite markers were unmapped in this analysis. Consequently, the mapping ratio of these markers is 78.6%. The female and male maps contained 480 and 417 markers, respectively, and both maps were found to have 21 linkage groups. The total length of the female map is 1,388.1 cM, with an average interval of 3.06 cM. Linkage group size ranged from 34.3 cM to 98.8 cM. The number of loci per genetic linkage group varied from 10 to 53. The male linkage map spanned a total genetic distance of 1480.9cM. The length of each linkage group varied from 41.5 to 90.8cM and contained 10–49 loci per group, with an average interval of 3.75 cM. Sex-specific genetic linkage maps are presented in Fig. 1 and Fig. 2.

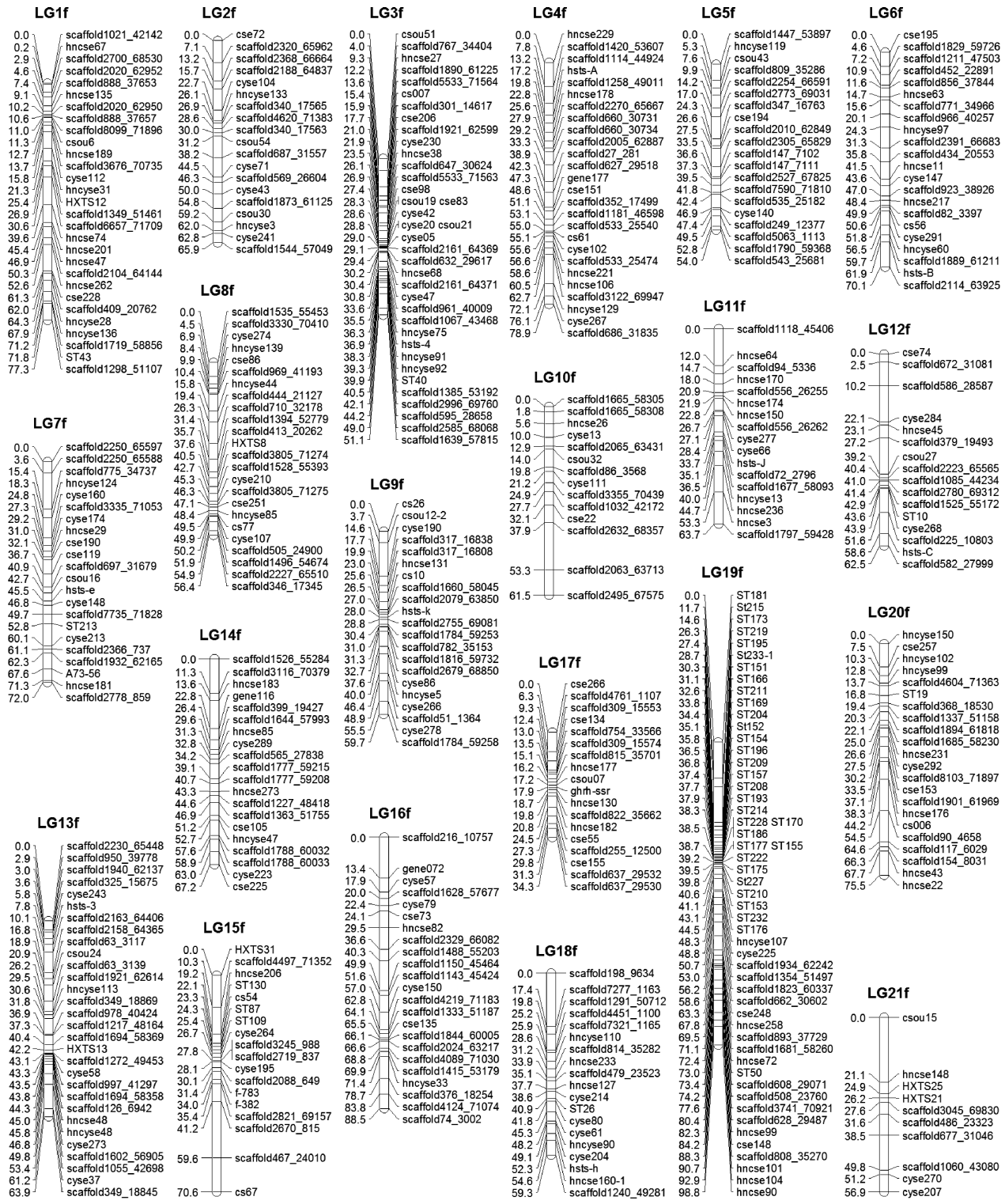

Fig. 1 Linkage maps of the female-specific map for *Cynoglossus semilaevis*

The female-specific genetic map comprises 480 markers assigned to 21 linkage groups (LG1f-LG21f), and spans a total map length of 1388.1cM. Genetic distances in Kosambi centimorgans are listed on the left side of the linkage groups, and markers are listed on the right side of the linkage groups.

The female and male maps display 474 and 417 unique positions, respectively. Estimated genome lengths, based on the two methods, were 1,516.8 cM (Ge1) and 1,529.0 cM (Ge2) for the female, and 1,638.4 cM (Ge1) and 1,659.9 cM (Ge2) for the male. The average of these two values was taken as the expected genome length, namely 1,522.9 cM for the female and 1,649.1 cM for the male. Based on recent estimations of map

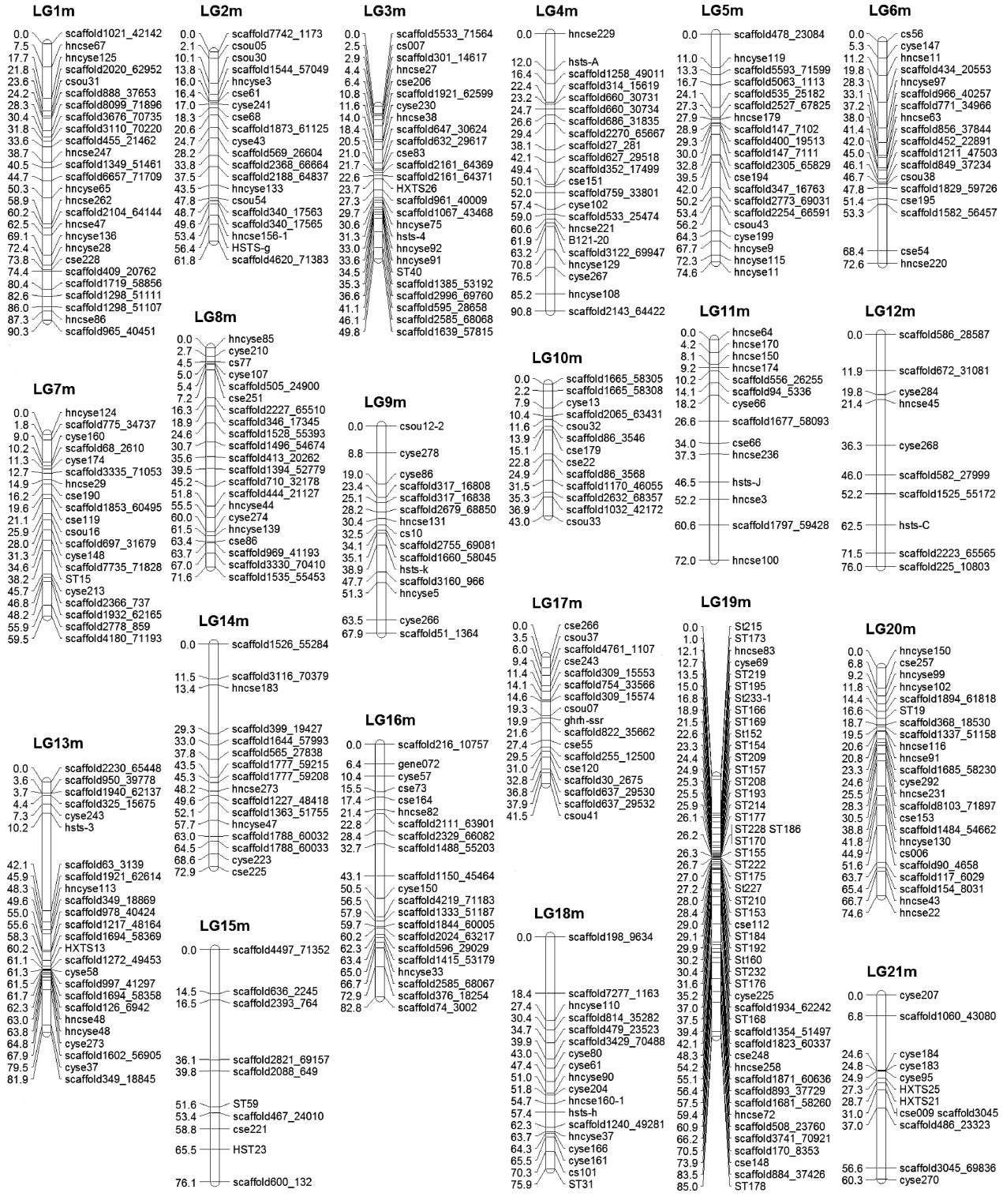

Fig. 2 Linkage maps of the male-specific map for *Cynoglossus semilaevis*

The male-specific genetic map comprises 417 markers assigned to 21 linkage groups (LG1m-LG21m), and spans a total map length of 1480.9cM. Genetic distances in Kosambi centimorgans are listed on the left side of the linkage groups, and markers are listed on the right side of the linkage groups.

length, the genomic coverage of the female and male maps was 91.1% and 89.8%, respectively. The characterization of genetic linkage maps of half-smooth tongue sole is presented in Table 1 and Table 2.

2.5 Integrated maps

Either bridge markers or homologous loci were used to identify co-linear regions in the female and male maps. The integrated map was composed of 565

Table 1 Number of markers and genetic length for each linkage group

LG	Female maps			Male maps			Integrated maps		
	No. of markers	Length (cM)	cM/marker	No. of markers	Length (cM)	cM/marker	No. of markers	Length (cM)	cM/marker
LG1	29	77.3	2.67	26	90.3	3.74	38	102.2	2.69
LG2	19	65.9	3.47	20	61.8	3.09	25	77.7	3.11
LG3	37/35 [a]	51.1	1.38/1.46 [b]	26	49.8	1.92	38/37 [a]	66.1	1.74/1.79 [b]
LG4	26	78.9	3.03	22	90.8	4.13	31/30 [a]	88.9	2.87/2.96 [b]
LG5	20	54	2.70	20	74.6	3.73	28	66.9	2.39
LG6	22	70.1	3.51	18	72.6	4.03	27	87.3	3.23
LG7	22	72	3.27	20	59.5	2.98	26	74.8	2.88
LG8	24	56.4	2.35	21	71.6	3.41	24	69.6	2.90
LG9	21	59.7	2.84	15	67.9	4.53	22	60	2.73
LG10	14	61.5	4.39	13	43	3.31	18	78.1	4.34
LG11	17	63.7	3.75	14	72	5.14	19	79.6	4.19
LG12	16	62.5	3.91	10	76	7.6	16	67.2	4.20
LG13	30	63.9	2.13	26	81.9	3.15	30	75	2.50
LG14	20	67.2	3.36	16	72.9	4.56	20	52.8	2.64
LG15	18/17 [a]	70.6	3.92/4.15 [b]	10	76.1	7.61	26/23 [a]	75.1	2.89/3.27 [b]
LG16	23	88.5	3.85	21	82.8	3.94	27	87.5	3.24
LG17	18	34.3	1.91	17	41.5	2.44	23	39.7	1.73
LG18	19	59.3	3.12	18	75.9	4.22	24	56.6	2.36
LG19	53/50 [a]	98.8	1.86/1.98 [b]	49	85	1.73	64/63 [a]	97.5	1.52/1.55 [b]
LG20	22	75.5	3.43	23	74.6	3.24	26	76.1	2.93
LG21	10	56.9	5.69	12/11 [a]	60.3	5.02/5.48 [b]	15	58.7	3.91
Total	480/474 [a]	1388.1	2.89/2.93 [b]	417/416 [a]	1480.9	3.55/3.56 [b]	567/561 [a]	1537.4	2.71/2.74 [b]

[a] Data are shown as number of markers mapped/unique locations. [b] Data are shown as centimorgans/marker and centimorgans/unique marker location.

Table 2 Summary of genetic linkage maps

	Female maps	Male maps	Integrated maps
Number of markers scored	613	568	720
Number of markers mapped	480	417	567
Average number of markers per group	23	20	27
Average marker spacing (cM)	3.06	3.75	2.85
Observed genome length (cM)	1388.1	1480.9	1537.4
Estimate genome length (cM)			
Ge1	1516.8	1638.4	1657.0
Ge2	1529.0	1659.9	1664.4
Ge	1522.9	1649.1	1660.7
Genome coverage (%)	91.1	89.8	92.6

microsatellite markers and two SCAR markers in 21 linkage groups (Fig. 3), covering a total of 1,537.4 cM with an average interval of 2.85cM. The genome length of half-smooth tongue sole was estimated to be 1660.7cM, and coverage of 92.6% was observed. The linkage group length varied from 39.7 cM to 102.2 cM, and the number of markers on the linkage group varied from 15 to 64 (Table 1 and Table 2).

2.6 Recombination rate

The availability of SSR markers in the male and female maps allowed an evaluation of the respective meiotic recombination rates. The recombination rates obtained from 21 linkage groups were on average 0.0306 in females and 0.0375 in males. Therefore, the relative recombination ratio (female-to-male; F/M) in these pairs was 1:1.2, slightly higher in males than females. The average recombination rate in integrated maps is approximately 0.029 in half-smooth tongue sole.

3 Discussion

Linkage analysis and map construction using molecular markers is more complicated in full-sib families of out-breeding species than in progenies derived from homozygous parents. For example, markers may vary in the number of segregating alleles, one or both parents

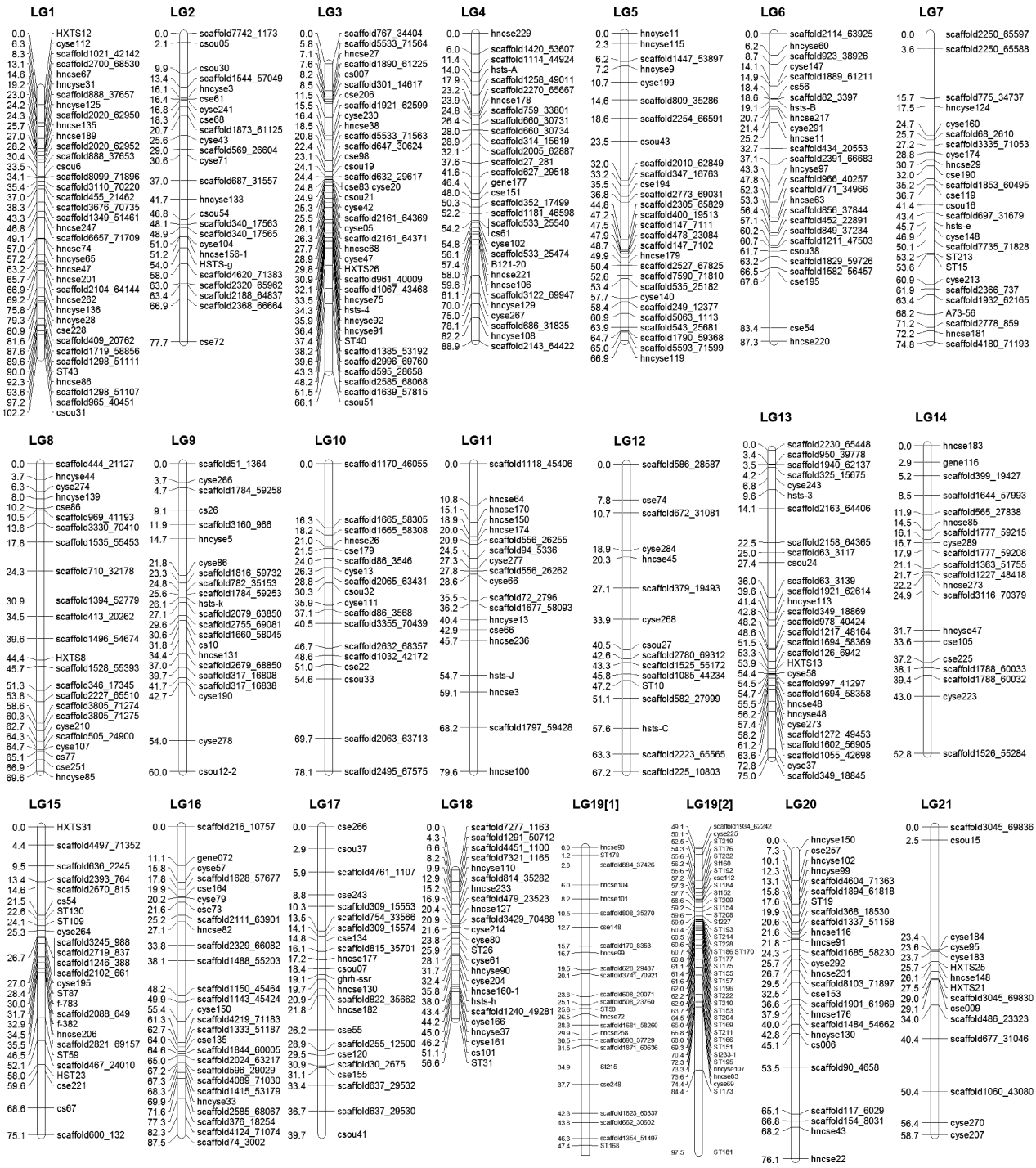

Fig. 3 Linkage maps of the integrated map for *Cynoglossus semilaevis*

The integrated map comprises 567 markers assigned to 21 linkage groups (LG1m-LG21m), and spans a total map length of 1537.4cM. Genetic distances in Kosambi centimorgans are listed on the left side of the linkage groups, and markers are listed on the right side of the linkage groups.

may be heterozygous, markers may be dominant or co-dominant, and usually the linkage phases of marker pairs are unknown. Given these differences, marker pairs provide different amounts of information for the estimation of recombination frequencies and the linkage phases of the markers in the two parents, and usually these have to be estimated simultaneously (Maliepaard et al., 1997). Therefore, the maps are constructed independently for maternal and paternal meiosis.

Genetic maps provide important genomic information and allow the exploration of QTL, which can be used to maximize the selection efficiency of target traits. The availability of a large number of genetic markers is essential for constructing a useful mid-density linkage

map and for QTL mapping of genetic traits of interest. In this study, we constructed a mid-density microsatellite genetic linkage map using 567 markers in half-smooth tongue sole. Both female and male maps were found to have 21 linkage groups, which is in agreement with the karyotype of $2n$=42. There were no small linkage groups (doublet or triplet), indicating that this linkage map is complete. Only 32 of the 720 markers studied remained unlinked to any other marker. This degree of completeness supports the utility of the genetic map as a reference tool for future genetic analysis in this species. In the mapping family, the male parent was derived from a wild population and the female selected from a cultured population, but the number of segregation markers in males (568) is less than that in females (613). This is possibly because the recombination ratio was higher in males than females or the number of genetic markers is restricted.

The marked sexual dimorphism of the growing half-smooth tongue sole has led to suggestions that the efficiency of the culture systems could be improved by setting up a production system focused on the faster-growing sex. In a previous study, a female-specific DNA marker f-382 was located on the linkage map of half-smooth tongue sole and this was the first report on the mapping of a sex-linked marker on a genetic linkage map in teleosts (Liao et al., 2009). We were able to map another SCAR marker f-783 onto the LG15f region in which female-specific SCAR marker f-382 was assigned. Both the male and female maps share the homologous region of LG15f containing the same microsatellite markers (scaffold4497_71352, scaffold2821_69157, scaffold467_24010), which imply that the LG15 segment is homologous in females and males, and is an indication of a pseudoautosomal region of the sex chromosome. The mapping of a sex-linked marker in a general population of half-smooth tongue sole is vital for the further development of mono-sex culture in this species. This is especially important in half-smooth tongue sole because of a large difference in the growth rate between males and females. The identified female-specific SCAR markers of f-783 and f-382 can be used for the molecular identification of genetic sex, and also provide an important tool for screening and isolation of the sex-determining locus and sex manipulation in half-smooth tongue sole.

The average interval between markers was slightly less for the female map (3.06 cM) than the male map (3.75 cM), suggesting that the recombination rate was slightly higher in males than in females. The recombination ratio between the male and female parents of half-smooth tongue sole was 1.2:1. Differences in map length can result from a variation in the number of recombination events in the two parents as well as variations in the number and location of the mapped loci. Despite this being a common phenomenon, the mechanism responsible for the different recombination rates between the genders is not well understood. Some studies have shown that recombination rate differences are associated with QTL (Kai et al., 2005). Selection using linked markers is more efficient when recombination does not occur between the markers and the QTL loci. It is common to find a difference in the recombination ratio between the two sexes in most aquatic species, but in these species, the recombination rate was higher in females than in males. For instance, the male/female recombination ratios are 1:3.25 in rainbow trout (Sakamoto et al., 2000), 1:8.26 in Atlantic salmon (Moen et al., 2004), 1:2 in halibut (Reid et al., 2007) and 1:1.43 in Japanese flounder (Castaño-Sánchez et al., 2010). The average recombination rate across all of linkage groups is approximately 0.029 in this study, which is higher than that in zebrafish (Shimoda et al., 1999), tilapia (Lee et al., 2005), catfish (Wang et al., 2007) and grass carp (Xia et al., 2010), and lower than rainbow trout (Rexroad et al., 2008), Asian sea bass (Wang et al., 2011) and Japanese flounder (Castaño-Sánchez et al., 2010).

In this mapping family, segregation distortion was observed for 165 markers and the distortion rate was approximately 23.0%, which is lower than the ratio of 33% reported by Liao et al. (2009). This suggests that a high ratio of segregation distortion may be a common phenomenon in half-smooth tongue sole. The distortion rate is 16% in channel catfish (Liu et al., 2003) and 16.3% in common carp (Cheng et al., 2009). The reasons for the distortion of the segregation ratios may be due to sampling errors (Plomion et al., 1995), scoring errors (Nikaido et al., 1999), the progeny population size and amplification of a single-sized fragment derived from several different genomic regions (Faris et al., 1998). Additionally, lethal effects caused by a recessive homozygote in the juvenile period may affect distorted segregation (Hubert *et al.*, 2004).

In conclusion, the second generation of the linkage map of half-smooth tongue sole containing 565 microsatellites and two SCARs has been constructed. With higher marker density (3.06 cM and 3.75 cM in the female and male maps, respectively), the new map is presently the densest flatfish linkage map. The map will not only facilitate selective breeding and mapping of

QTL, but also provide new data for comparative genomic studies.

Acknowledgements This work was supported by grants from Special Fund for Agro-scientific Research in the Public Interest (200903046) of China, National Nature Science Foundation of China (31130057, 31072202), and Taishan Scholar Project Fund of Shandong of China. We thank Jian Wu from the Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences for advice during data analysis.

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