A Detailed Linkage Map of Medaka, *Oryzias latipes*: Comparative Genomics and Genome Evolution

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ABSTRACT

We mapped 633 markers (488 AFLPs, 28 RAPDs, 34 IRSs, 75 ESTs, 4 STSs, and 4 phenotypic markers) for the Medaka *Oryzias latipes*, a teleost fish of the order Beloniformes. Linkage was determined using a reference typing DNA panel from 39 cell lines derived from backcross progeny. This panel provided unlimited DNA for the accumulation of mapping data. The total map length of Medaka was 1354.5 cM and 24 linkage groups were detected, corresponding to the haploid chromosome number of the organism. Thirteen to 49 markers for each linkage group were obtained. Conserved synteny between Medaka and zebrafish was observed for 2 independent linkage groups. Unlike zebrafish, however, the Medaka linkage map showed obvious restriction of recombination on the linkage group containing the male-determining region (*Y*) locus compared to the autosomal chromosomes.

GENETIC linkage maps using markers, such as phenotypic traits and expressed sequence tagged sites (ESTs), and anonymous DNA markers, such as random amplification of polymorphic DNA markers (RAPDs), amplified fragment length polymorphic markers (AFLPs), and microsatellites, are very effective tools for analyzing complex biological phenomena. Genetic maps with hundreds and thousands of mapped loci have been reported for various organisms (Postlethwait *et al.* 1994, 1998; Wada *et al.* 1995; Dib *et al.*1997; Dietrich *et al.* 1997; Knapik *et al.* 1998; Young *et al.* 1998). These maps have been used for various kinds of biological analyses, such as position-based cloning (Dietrich *et al.* 1997), quantitative trait locus analysis (Lander and Botstein 1989), comparative vertebrate genomics (Amores *et al.* 1998; Postlethwait *et al.* 1998), and detection of radiation-induced DNA mutations (Shimada and Shima 1997).

Medaka is a small freshwater fish native to Japan, Korea, and China (Yamamoto 1975; Naruse *et al.* 1994). This fish has been used widely as an experimental animal because of its relatively short life cycle, high fecundity, transparent egg chorion, small size, and availability of several inbred lines (Naruse *et al.* 1994). Medaka, zebrafish, Xiphophorus fish, and rainbow trout are members of the orders Beloniformes, Cypriniformes, Cyprinodontiformes, and Salmoniformes, respectively, distributed in relatively different taxonomic positions in teleost phylogeny (Nelson 1994; Naruse 1996). Comparative genomics of these fish together with progression in the mammalian genome project should facilitate studies of the evolution of genome structure and function in vertebrates.

A Medaka linkage map was first described by Aida (1921). He demonstrated that the male-determining factor (*Y*) was linked with the gene that controls carotinoid deposition in xanthophores (*R*). Since Aida's study, over 60 visible mutants have been isolated and analyzed for linkage (Tomita 1975, 1982), and a multipoint linkage map including 170 loci and 28 linkage groups has been established using RAPD fingerprints and allozyme analysis (Wada *et al.* 1995). As the Medaka haploid chromosome number is 24, this map has 4 excess linkage groups, indicating that at least four gaps remain to be filled in the map. The purpose of this study is to fill these gaps and to map expressed genes to compare the arrangement of orthologous genes among different species.

MATERIALS AND METHODS

Strains and genetic crosses: The AA2 (Shimada and Shima 1997) and HNI (Hyodo-Taguchi and Sakaizumi 1993)

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TABLE 1

Nomenclature of loci by combination of selective primers for AFLP analysis

*Mse*I selective primer	*Eco*RI selective primer							
	M-CAA	M-CAC	M-CAG	M-CAT	M-CTA	M-CTC	M-CTG	M-CTT
E-AAC	EM1	EM2	EM3	EM4	EM5	EM6	EM7	EM8
E-AAG	EM9	EM10	EM11	EM12	EM13	EM14	EM15	EM16
E-ACA	EM17	EM18	EM19	EM20	EM21	EM22	EM23	EM24
E-ACT	EM25	EM26	EM27	EM28	EM29	EM30	EM31	EM32
E-ACC	EM33	EM34	EM35	EM36	EM37	EM38	EM39	EM40
E-ACG	EM41	EM42	EM43	EM44	EM45	EM46	EM47	EM48
E-AGC	EM49	EM50	EM51	EM52	EM53	EM54	EM55	EM56
E-AGG	EM57	EM58	EM59	EM60	EM61	EM62	EM63	EM64

E and M indicate the sequences GACTGCGTACCAATTC and GATGAGTCCTGAGTAA, respectively.

strains are inbred strains established from Southern and Northern Medaka populations, respectively (Sakaizumi 1986). In the phenotypic trait loci, the genotypes of AA2 strain are *b*/*b*, *lf*/*lf*, and *gu*/*gu* and those of HNI strain are wild types. These two populations are genetically divergent and polymorphisms between them are identified easily (Naruse *et al.* 1994; Matsuda *et al.* 1997a,b). The sequence average divergence between AA2 and HNI strains was ~0.8% in the coding regions and 2.6% in the intron regions. The insertions or deletions of nucleotides were also observed in the intron regions. By crossing AA2 female and (AA2 female × HNI male) F_1 male, 39 backcross progeny were obtained for genotyping.

Establishment of cell lines for a reference mapping panel: A caudal fin was taken from each of the backcross progeny to establish the cell lines. The fins were sterilized with Dakin's solution, washed twice with PBS, and put into L-15 medium supplemented with 20% FBS at 33° (Komura *et al.* 1988). The remainder of each body was fixed in 100% ethanol.

Genomic DNA extraction: Genomic DNA was extracted from the cultured backcross cell lines and the ethanol fixed bodies by proteinase K digestion followed by phenol-chloroform extraction and isopropanol precipitation.

Markers: A total of 634 markers, including AFLPs, RAPDs, ESTs, internal repeat sequences (IRSs), sequence tagged sites (STSs), and 4 phenotypic markers, were used to establish the genetic linkage map.

Phenotypic markers: Four phenotypic markers, *Y* (male-determining gene), *b* (colorless melanophore), *lf* (leucophore free), and *gu* (guanineless), were used. Recessive mutant phenotypes for the *b*, *gu*, or *lf* loci have been described by Tomita (1982) and Wada *et al.* (1998). At 2–3 months of age, the adult fish were sexed based on gonadal morphology and sexual dimorphism of the dorsal and anal fin sharp.

AFLP marker: AFLP marker analysis (Vos *et al.* 1995) was performed using the AFLP analysis kit (GIBCO-BRL, Gaithersburg, MD) following the manufacturer's instructions. The AFLP markers were named using the enzymes and selective primer sets used and the product size. For example, EM8-1 was the largest AFLP band obtained using *Eco*RI and *Mse*I and E-ACC and M-CTT selective primers. The AFLP markers identified after the initial survey of AFLPs were given an additional alphabetical identifier, such as EM8-d. The nomenclature for each locus using the combination of selective primers is shown in Table 1.

EST and STS markers: To assign the loci encoding expressed genes to each linkage group, we used polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis. Primers were designed on the basis of cDNA sequences previously described by others or sequences determined by us. PCR amplification of the genomic counterpart of the cDNA was carried out using LA-*Taq* DNA polymerase (Takara, Kyoto, Japan), using genomic DNA from the AA2, HNI, and F_1 strains as template. Amplifications were carried out in a PCR thermal cycler MP (Takara model TP-3000) as follows: denaturation at 95° for 5 min, followed by 30 amplification cycles of 98° for 20 sec, 55° for 40 sec, and 72° for 2 min, and a final extension of 72° for 5 min. When multiple bands were observed after amplification, the annealing temperature was increased to 60° or 65°. The amplified fragments were sequenced and analyzed for restriction sites, insertions/deletions, and allele-specific polymorphism. The fragments amplified from the genomic DNA of each backcross progeny were digested with the appropriate restriction enzymes, separated on a 12% slab polyacrylamide gel (Davis 1964) or 3% agarose gel, and stained with ethidium bromide. Table 2 lists the genes mapped in the present study. Most EST and STS markers except the *Hox* genes were genotyped on a 12% slab polyacrylamide gel. All *Hox* genes except *Hoxa9a* were genotyped on a 3% agarose gel. The mappings of the *Hoxa4a* and *Hoxa5a* genes and the *Hoxc4a* and *Hoxc5a* genes were done using the polymorphism on the intergenic regions between two genes. Thus, the same primer sequences were listed in Table 2. The *UAA* and *UBA* genes and *Ki-ras* gene and *NK-1* marker could be amplified with the same primers. Therefore, the same sequences were listed in Table 2. For the amplification of the *Bf*/*C2* gene, the nested PCR using two sets of primers was used. The same PCR conditions were used for the genotyping of the STS markers.

RAPD and IRS markers: PCR amplification of RAPD markers was essentially the same as previously described (Kubota *et al.* 1995; Wada *et al.* 1995). Primers to amplify the intersequence between two short interspersed repetitive elements (Naruse *et al.* 1992; Shimoda *et al.* 1996; Uchiyama *et al.* 1996) were used for IRS fingerprinting (McCarthy *et al.* 1995). Primer sequences for RAPD and IRS fingerprinting are shown in Table 3. The locus designated IRS-AB is a marker obtained by PCR using the IRS-A and B primers. PCR amplification incorporating [^{32}P]dCTP for IRS fingerprinting was carried out using LA-*Taq* DNA polymerase (Takara, Kyoto, Japan) and the same conditions were used for EST and STS amplification. The PCR products were electrophoresed in a 5% urea long-range sequencing gel (FMC Bioproducts, Rockland, ME) and were visualized by autoradiography.

Linkage analysis: Segregation of the markers was analyzed using a reference mapping panel and MAPMAKER Macintosh version 2 (Lander *et al.* 1987; formatted for Macintosh by Dr. Tingy, Dupont Corp., Wilmington, DE). A minimum LOD

TABLE 2

List of EST and STS markers mapped by PCR-RFLP

No.	Gene	LG	Primer forward	Primer reverse	Polymorphism	DDBJ/GenBank accession no.
1	Act-c	8	AGCCGAGAGGGAAATTGTCCGT	CAACTGCAGATGCCTGGGGTG	*Msp*I	D89627
2	Act-m	3	CTGCTGAGCGTGAGATCGTG	GTTTTCACAGCCTCTGTCTCCG	*Fok*I	D87740
3	B2m	23	ATGAAAGAGCTTTTCTTCATTGC	CTGGCCAGGGTCATGACTGTACAC	in/del	AB006593
4	Bf/C2	14	GCTCAACATCTACATTCGCCT CCTCTTTGGAAACAGAC	TGACAACTTCATACACATCA TTCACAGCTCAGCAGGT	*Rsa*I	D84063
5	Blue	5	TCCTGTGTGTCGAAGGCCTCCACA	GACTTTTGAGACTTCAGTGGTTGA	in/del	AB001602
6	C3-1	1	GCAGAGATGCTTTGAAT	ATTTTATTCTGCACACATCTTTTACCA	*Hin*fI	AB025575
7	C3-2	1	GCAGAGATGCTTTGAAT	TTTCCACTGGGCCTTGGTAAAAT	*Hph*I	AB025576
8	C4	16	TGTGCAGAAAGACCCTGCTATAAGG	TGGTGTTATGGCGGAGGCAAG	in/del	AB025577
9	Casp3A	10	TGATATACGGCACTGATGGTCCGG	GGTGCTCCTCCACGAGTAATAGCC	*Nla*III	AB032609
10	Casp3B	1	CAGATTAAACAGCTTTTGACTGATGGT	TGACTCAAAAGGATGCAGACGA	in/del	AB032608
11	DAB	18	GTCTGGAGAGAAAATCTCCTG	TCTGACTCTGGCATGGACGGGT	in/del	AB033212
12	dd001	22	CCATCAACACCAGCAGTAATCA	GGACAGGTTGTGTTTTTGAAATTTGC	*Eco*RI	AB033201
13	dd004	7	CAGTCACAGCAAGGCTCTCC	GTCCAAAGTGCACGACTTCC	in/del	AB032765
14	dd007	6	CTCCCCTCTTAAGGCAAAGATG	CAGTTTATCCCGTCTCAACTG	*Mnl*I	AB033202
15	dd009	22	CCACAGACCCAAGACTGGGC	CAGCTGATGAATGGTAATGC	*Msp*I	AB033203
16	dd024	7	GTTCCTGTCAGCAGCGTATTT	AGCCATAAAGAAGAACAACCCA	*Fok*I	AB033204
17	dd039	15	TGGGAGTGGTGTAGGACTTCTT	TGTTAAACGAAGGGGCTTGTTAG	in/del	AB033205
18	dd048	1	ACGGTTCTTCTCCTGTGGTCTA	AACAATACAAACTGAGACCCTGC	Allele specific	AB033206
19	Ef-1A	11	AATGTGGAGTCCTATCAG	AGGACACGTGTTACTCCCAC	in/del	AB020734
20	Eya3	11	CCAACTGTTTGATTTACGTGTTCACGCA	AAGCCATCAGCCAGGAAGTTGTAGTT	Allele specific	AB032898
21	Fgfr2	15	AAGGACAAACCCAAGGAGGCTGCT	TTGTTCGTCTGAGACACGAGCAATG	in/del	D13551
22	Fgfr3	22	TAAGGAGAAGCCCAATAAGCCACTG	CTGCTCATCTGGAATTTTGCAGGTG	*Ava*II	D13552
23	Fgfr4	14	CAAGGACGGCCCGGAGCAGGCCACC	CTGTTCCTCTGGCACTTTGGTCACA	*Hin*fI	D13553
24	Green	5	CTTCTTTTCAAAGAGCTCAGCCC	ACAGGCTATCATAGTCGAGGCTG	in/del	AB001603
25	Hoxa10a	11	CCTCTTCAACATGTACCTGACCCGAG	TTGGACCCCACAGTGGAAATGAGTCATATT	Allele specific	AB026974
26	Hoxa1a	11	CCATTTCAACAAGTACCTGACTCGGGCT	GCACACGATTGACCCGTAAACGTCTC	*Psp*1406 I	AB026948
27	Hoxa3a	11	GTACGCCATGCCAACGTCATATCCAC	AAGGTGGGAGCCTTGAGGAGGCGCA	Allele specific	AB026951
28	Hoxa4a	11	CCACTTTAACCGGTATCTGACCCGCA	CAAGAGTGTAAAATACTGAAGATCGGTCCATC	Allele specific	AB026954
29	Hoxa5a	11	CCACTTTAACCGGTATCTGACCCGCA	CAAGAGTGTAAAATACTGAAGATCGGTCCATC	Allele specific	AB026961
30	Hoxa9a	11	GCATCACCAAGAGAGAAGGACGAGCTG	CACTCGCGTGGAGCCAGTTGGAAAC	*Hin*P1 I	AB026972
31	Hoxa9b	16	CTTTTGTGAATATCCCGTGCGAGTCCTC	TGGTGGGCTTCGCTTGCAGCCAAC	Allele specific	AB026971
32	Hoxb1b	19	CCACTTCAACAAGTACCTGACCCGC	GTGCAAACAATGACACCAACTCAAATCACAGC	*Hae*III	AB026947
33	Hoxb4a	8	TCACTACAACAGATACCTGACGCGCAGGC	ACGGACGTGAGAAGGAATCGCTTCGATCC	*Msp*I	AB026956
34	Hoxb5a	8	CCACTTCAATCGCTACCTGACCAGGA	CAATGGCATGCTGAAAAGTGCTGCGC	Allele specific	AB026959
35	Hoxb5b	19	CCTTGGATGCGGAAACTGCACATAAGC	AGCGTCTGGTAGCGGGTGTACGCAG	*Dde*I	AB026962
36	Hoxb6b	19	CCACTTCAATCGCTACCTGACCAGGA	CAATGGCATGCTGAAAAGTGCTGCGC	Allele specific	AB026964
37	Hoxb9a	8	GAGTAAGGAGGGATGTGAAGGCAGC	CGCGCGTGCAGCCAGTTGGCTGAAG	*Xba*I	AB026969
38	Hoxc10a	7	CTTGTTCAACATGTATCTGTCTCGGGAGC	ACAACGATAGTGCCGCACTGTGCAG	*Hin*P1 I	AB026973
39	Hoxc3a	7	CCACTTCAGCCCTTACCTTTGTCGAC	CAACAATGATATGAAAAACCTGCTGGATG	*Dra*I	AB026953
40	Hoxc4a	7	TCACTTCAACCGGTACCTGAGCCGC	CTTAAAGGAAACCGCACAAGATAGCTACG	Allele specific	AB026955
41	Hoxc5a	7	TCACTTCAACCGGTACCTGAGCCGC	CTTAAAGGAAACCGCACAAGATAGCTACG	Allele specific	AB026960
42	Hoxc9a	7	TCCAGAGTCGGAGCTGCTGACTTCG	CGTCGAACGAGCATGTATCCAATTTGCC	*Hin*dIII	AB026970
43	Hoxd3a	21	CTGACGCATTTGTAATATATGGTTCCG	GAAAAGAGCGCAACATAATTTTGGCAC	in/del	AB026952

(*continued*)

TABLE 2

(Continued)

No.	Gene	LG	Primer forward	Primer reverse	Polymorphism	DDBJ/GenBank accession no.
44	Hoxd4b	15	CCACTTCAGCCGCTACCTCACGCG	CTCTTACAGTGAGAGTTACAGCTTTCCC	*Bbv*I	AB026957
45	Hoxd9a	21	GCAGCGCAGAGCAAGCGAGATTTCG	GAGCGCGCGTGGATCCAGTTTGCAG	*Mbo*II	AB026967
46	Hoxd9b	15	CCTCTACAACATGTACTTGACCCGG	CCGGCTTTGAAACATGCAGGAGGACGAG	*Nla*III	AB026968
47	Hsc70	14	ATGCCAGAGGGAATGCCAGGAG	TTCAGCATTTATTGTAGAGTGACATC	*Dra*I	D13669
48	Ki-ras	6	ATGACGGAATATAAGCTGGTGGTG	GAGGAAGCCCTCCGCTGTCCT	in/del	AF030545
49	LMP2	11	GGCTCTGATTCCAGAGTGTCTGCA	CAGAGTGGCAGCTGAGCGAACCT	*Bgl*II	D89724
50	LMP7	11	CTGTAYGRCTGAGAAACAA	TCCTTGTCCCATCCACAGAT	*Pvu*II	D89725
51	mfOR2		ATGTTGATACTGTCCTGGCTTTGG	AATAATCTCAGTGGTTGGTGTCCTT	*Taq*I	AB022647
52	mfOR3	14	CTGAAATGACCCCACCCTTGCA	TGCAGAGGAAACATGATAGC	in/del	AB022648
53	mfOR4	14	TCAGAGGCATCCAAGTATCCAG	GTACTCAGGGAAACGATGTAGC	*Mnl*I	AB022649
54	Msx10	15	AGACCAAGTGCGAGTCACCGAACG	GATGGAGAGGTACTGTTTCTGACG	In/del	AB033287
55	Msx4	14	ACCGAAGGAAGACTTGATGGCAGT	GCATGGACAGGTACTGCTTCTGCC	*Mnl*I	AB028880
56	Olgc1	12	GGCCGAGCCGTGACTTTATTTGTTCTAG	GAGGGTGCTTTGCTCCACAGTTACACAA	in/del	AB004921
57	Olgc2	16	CAGCAGAGGGTCGTAAGAGG	CGTCTTTATTTATTCACTCCTTCAG	in/del	AB016082
58	Olgc3	2	CTCTTGCTGATTTCTGAAC	TCAGCGTCACTTCGTAAC	*Hin*fI	AB000899
59	Olgc4	13	CAACGGTGCTAATGTAATGAAAGT	CTCAGACATATTGGGGGAAAGTAA	*Fok*I	AB000900
60	Olgc5	14	CTCAGCAGTGCCACAATAAACAATGACTCC	GAACATAAATCGCTGGAGACTGACATGGGA	in/del	AB000901
61	Pax6	3	CCCTGTAACCACCCACTCCACCATC	AACACCGGGCACTTTACAGAAGGTCCA	in/del	AB002408
62	Phr	17	GAYAAYTTYTGCTTCTACAAC	CATGGANGCCYCAGATNGACCACAT	in/del	D26022
63	Red	5	TGGAGTGCAGTCCTACATGATTG	CTGTCTTTGATGTAGAAACTTCAG	*Nla*III	AB001604
64	Rhodopsin	7	AAACACCACCGAGGGGCTGATAG	ATCCAGGTGAAGACCAAACCCAT	*Ehe*I	AB001606
65	Shh	20	ACCACTTCGAGGAGTCCCTTCAC	TGTTCCCTCTCCCTTCTTGATGA	*Rsa*I	AB007129
66	Six3	19	ACCCCGACTCAAGTAGGGAACTG	CCAGCATGATATGGGATAGATCCAG	*Nla*III	AJ000937
67	Tap2	11	AAATTCTTCTGGACGGGAATCCA	TCACCACAAGAGTCTGGTTTGGG	in/del	AB033382
68	Tp53	18	AGGTGTCCCCACCACCAAAC	AGTGAGGATGGTGAGGATGGG	in/del	U57306
69	Trp1	18	ATGCCTTCCTTAATGATGTGTTG	ACCCGCACGGGTCGTCAACG	*Mnl*I	AF072305
70	Tyr	13	TACTACGTGTCCAGAGACACCTTC	AGTGGACCTTCTGCAGTAGCGTTAC	*Dra*I	AB010101
71	UAA	11	TCCATCAGTGTCTCTCCTCCAGAAG	GCAGACACAGAGAGAATGACAGCG	in/del	AB026977
72	UBA	11	TCCATCAGTGTCTCTCCTCCAGAAG	GCAGACACAGAGAGAATGACAGCG	in/del	AB026978
73	UCA	22	TATTTTTGTTGCTGTCATTG	AGCCTAAATCACAAAAGGGGTTT	Allele specific	AB033381
74	Violet	23	GAACTTTGTCGTTCTTCTGGCGA	ACTTCCTTCTCAGCCTTCTGCGT	*Bss*SI	AB001605
75	Yc-1	1	GAGGTAATAGTTGGCAAAGGAGC	TACTGAGCTCAACCAGAGGACA	*Sau*3A1	AB033606
76	Yc-2	1	AAGCTGCGCACCTTCTATGAGAAG C	ACTTGCGCTTGCGCTCACCAGT	*Rsa*I	AB033607
77	NK-1	8	ATGACGGAATATAAGCTGGTGGTG	GAGGAAGCCCTCCGCTGTCCT	in/del	[a]
78	FS-1	19	GAATTCAAGCCAAAGATG	TTAACTGAAACAAAATGAAGG	Allele specific	AB033330
79	OPH3-1	12	ACTCTGTCAGTCTGGATCTG	ATGCTGATAACATGAGGTGC	Allele specific	AB033329
80	Can3	7	GAAACTAGTGCTGACAAGCAAGGC	GAGCTGTGGAAGTCCCTCCATTT	in/del	AB033286

Act-c, cytosolic β actin; Act-m, muscle actin; B2m β2-microglobulin; Bf/C2, complement factor B/C2; Blue, blue visual pigment; C3-1 and C3-2, complement C3; C4, complement C4; Casp3A and Casp3B, caspase 3; DAB, MHC class II B; Ef-1A, elongation factor 1α; Eya, eyes absent; Fgfr, fibroblast growth factor receptor; Green, green visual pigment; Hox, homeobox gene; Hsc70, 70-kD heat-shock protein cognate; Ki-ras, ki-ras proto-oncogene, LMP, low-molecular-weight polypeptide; mfOR, olfactory receptor; Msx, msh/msx class homeobox; Olgc, guanylyl cyclase; Pax6, paired box homeotic gene; Phr, photolyase; Red, red visual pigment; Rhodopsin, rhodopsine; Shh, sonic hedgehog; Six3, sine oculis related gene 3; Tap, transport associated protein; Tp53, tumor suppressor protein p53; Trp1, tryrosinase related protein 1; Tyr, tyrosinase; UAA, UBA, and UCA, MHC class I A; Violet, violet visual pigment. Yc-1and Yc-2 are the expressed sequence tagged site markers located on the sex chromosome.

[a] NK-1 is a marker that is amplified under low annealing temperature (55°) but not amplified under high annealing temperature (60°) using primers for Ki-ras. FS-1 and OPH3-1 are anonymous sequence tagged site markers. Can3 is a simple sequence repeat (CA repeat) marker.

TABLE 3

Primers used for RAPD and IRS marker analysis

Primers	Sequence	Length (mer)
DxIR	ATGCCGTCCTTTATCTAGAAC	21
HSP7	CGATTTGATGATCATGTTGTACAGT	25
HSP8	CTTCATCAGGATTTATGATCTTGTT	25
LCF	ATGGACCTGCTGGCCAAAGCA	21
M3	CCTGTCGAAAGGGAGTCAAATTAT	24
pBR	ATGCAGGAGTCGCAT	15
T7	GCATAATACGACTCACTATA	20
TyrA	ATGTTCTTGGCTGTTTTGTA	20
U42	TTCCATACCTGGGAACGAGT	20
IRS-A	TGGCGACCCCTGATGGGAGAA	21
IRS-B	CAGCACGAGCTAAACTGGGCT	21
IRS-C	TGGGGTCATCTAAGATAGCTC	21
IRS-D	TCAGAGCACTGTCTAGTGGGT	21
IRS-E	GTCATNGTAGGGAGAACANGTCAATG	26
IRS-F	CCACGGATGCACGAGGAGAACACAC	25
IRS-G	GCACACGCATACACAGACATGCACACAC	28

score of 3.5 and maximum θ of 0.35 were used for grouping the markers. Marker orders were modified after visual analysis of the distribution of marker genotypes in the reference mapping panel.

RESULTS

Medaka linkage map: Linkage analysis of 634 markers, including 488 AFLPs, 76 ESTs, 28 RAPDs, 34 IRSs, 4 STSs, and 4 phenotypic markers, was used to construct a genetic map with 24 linkage groups corresponding to the haploid number of Medaka chromosomes (Figure 1). One EST marker (mfOR2) was not linked with any other markers under the linkage criteria used in the present study. The cumulative map length was 1354.5 cM. Table 4 shows the cumulative map length and number of loci in each linkage group. The previous map (Wada *et al.* 1995) consists of 170 markers in which 96 loci showed recombination in 20–100 meioses. The present map consists of 633 markers in which 258 loci showed recombination in 39 meioses. In comparison between the present and previous maps, the density of informative loci in the present map is 2.6 times higher than that in the previous map.

Of 76 EST loci, 9 were unidentified expressed genes cloned by differential display to detect mRNA sequence differences between the HNI and AA2 strains. We have described these unidentified expressed genes as dd001 to dd0048. The genes *Yc-1* and *Yc-2* were identified initially as sex-linked markers (H. Wada, H. Mitani and A. Shima, unpublished results).

We renamed each linkage group according to the number of the AFLP, RAPD, and IRS markers for the following reasons. First, because the anonymous DNA markers were expected to be randomly distributed throughout the genome and because the number of markers on each linkage group may reflect the physical size of each chromosome, the order of the linkage groups may reflect the order of chromosome size. Second, 11 linkage groups (II, VIII, X, XIV, XIX, XXIII, XXIV, XXV, XXVI, XXVII, and XXVIII) previously published contained fewer than three loci and the correspondence of LGVII, IX, X, XIII, XXII, XXIV, XXVI, XXVII, and XXVIII to the linkage groups in the current map could not be determined. To reduce the possible confusion of the linkage group names we used arabic numerals instead of the roman numerals for the name of each linkage group. The names of the current linkage groups (LG) refer to the previous linkage groups (Wada *et al.* 1995) as follows: LG1, I; LG2, III; LG3, XII; LG4, V; LG5, VI; LG6, XI; LG8, XVII; LG12, II; LG13, XVIII; LG14, XX; LG15, XXI; LG16, IV; LG18, XIV; LG19, XVI; LG20, VIII; LG21, XV; LG22, XXIII; LG23, XXV; and LG24, XIX. Linkage groups LG7, LG9, LG10, LG11, and LG17 were newly identified linkage groups in the present study.

Linkage relationships: Ten kinds of gene families were mapped in the present study: actin, caspase, fibroblast growth factor receptor, opsin (visual pigment), guanylyl cyclase, msx/msh type homeobox, hox, complement C3 and C4, olfactory receptor, and immunoglobulin super gene families. The members of the actin, caspase, fibroblast growth factor receptor, and guanylyl cyclase gene families were not linked. The *red*, *green*, and *blue visual pigment* genes were mapped to LG5. The *violet* and *rhodopsin* genes were located on LG23 and LG7, respectively. The *Bf/C2*, *Msx/msh related gene* (*Msx4*), *hsc70*, *mfOR3*, *mfOR4*, and *Fgfr4* loci mapped to LG14. Linkage was detected between the major histocompatibility (MHC) class I A genes (*UAA* and *UBA*), *LMP2*, *LMP7*, *Tap2*, *HoxA* cluster, and the *Ef-1A* genes (LG11). The third MHC class I A gene, *UCA*, mapped to LG22. Twenty-two *Hox* genes mapped to seven different

linkage groups (LG7, LG8, LG111, LG15, LG16, LG19, and LG21).

Linkage group 1 had a total map length of 44.2 cM, and 49 mapped loci. The male-determining factor, *Y*, was mapped to LG1, and, as previously reported, a phenotypic trait marker, *lf* locus (Wada *et al.* 1995, 1998), was mapped to LG1 in the present study. The *R* locus has been reported to be linked with the *Y* locus (Aida 1921). In addition to these loci, we have placed six EST markers, the *caspase 3B* gene, two *C3* genes (Kuroda *et al.* 2000), and three unidentified expressed genes (*Yc-1*, *Yc-2*, and *dd048*) on LG1. These loci were located on the *X* and *Y* chromosomes and genetic recombination was observed among the markers on LG1.

DISCUSSION

Establishment of cell lines for a reference mapping panel: The accumulation of mapping data using the same mapping panel is very important for eliminating linkage relationship ambiguities among markers. Because the body of Medaka is small, the amount of DNA from each individual could be a limiting factor for data accumulation. To overcome this, we established 39 permanently growing cell lines from backcross progeny obtained by crossing AA2 female with (AA2 × HNI) F_1 male. Thus, DNA from these cell lines could be used, even for techniques such as RFLP analysis by Southern blotting, without exhausting the reserve of genomic DNA. Possible genetic alternations during cell subculture could be checked using DNA extracted from the remaining bodies or another typing panel. In our case, the data obtained from the cell lines were consistent with the typing data from another panel (Kuroda *et al.* 1996; Namikawa-Yamada *et al.* 1997; data not shown).

Medaka linkage map: The total map length in the present study was 1354.5 cM, while the previous map had a total length of 2480 cM (Wada *et al.* 1995). This difference may be due to an overestimation of interlocus map distance in the previous map resulting from distance calculation errors caused by the use of two typing panels. For example, four gaps of >20 cM and one linkage group consisting of only three loci and spanning 24 cM were present in the previous map (Wada *et al.* 1995). In the present study, we used only one mapping panel to minimize data calculation errors.

In this study, only 1 (mfOR2; Sun *et al.* 1999) of the 80 EST and STS markers was not linked to any other marker, indicating the present linkage map provides reasonably good coverage of the Medaka genome. The number of linkage groups was 24, the same number as Medaka's haploid chromosome number. Correspondence between the linkage group and the chromosome can be clarified by the identification of a marker near the centromere, because each chromosome has only one centromere. We are now trying to identify centromeric markers using gene-centromere mapping by diploid gynogenesis (Naruse *et al.* 1988; Naruse and Shima 1989; Johnson *et al.* 1996). To date we have found centromeric markers for LG1, LG8, LG12, LG14, and LG23 (data not shown). These 5 linkage groups correspond to five different chromosomes.

MHC gene organization: Two MHC class I A genes (*UAA* and *UBA*) were linked to each other and were assigned to the same linkage group (LG11) as the *LMP2*, *LMP7* (Namikawa-Yamada *et al.* 1997), and *Tap2* genes. The MHC class II B (*DAB*) locus was mapped to LG18 and the *B2m* gene was mapped to LG23. No linkage was observed among the MHC *class I A*, MHC *class II B*, *complement Bf/C2*, *C3*, and *C4* genes. These results indicate that the Medaka counterparts of the mammalian MHC genes are dispersed throughout the Medaka genome, except for the cluster of the MHC *class I A* gene and the genes involved in class I antigen presentation. A similar result has been reported in zebrafish (Bingulac-Popovic *et al.* 1997) and rainbow trout (Hansen *et al.* 1999), suggesting that a common euteleostei ancestor may have had a dispersed MHC organization. Whether the dispersed MHC is ancestral to, or derived from, the mammalian-type centralized MHC has yet to be determined; however, the genes involved in class I antigen presentation appear to constitute the core of the MHC.

Hox gene organization: Mammals have ~40 *Hox* genes organized into four clusters on different chromosomes (Scott 1992). Although the pufferfish has four *Hox* clusters (Aparicio *et al.* 1997), recent studies of the *Hox* cluster organization of zebrafish, *Danio rerio*, show that the zebrafish has at least seven *Hox* clusters, suggesting a *Hox* cluster duplication in an ancestor of the zebrafish lineage (Amores *et al.* 1998). In the present study, 22 *Hox* genes were mapped to seven different linkage groups in Medaka. This indicates that Medaka has at least seven *Hox* clusters and suggests that this fish may also have additional duplications of the *Hox* clusters. Medaka, pufferfish, and zebrafish are members of Beloniformes in Acanthopterigii, Tetranodon in Acanthopterigii, and Cypriniformes in Ostariophysi, respectively (Nelson 1994). Medaka and zebrafish are distributed in largely different taxonomic positions in teleost phylogeny (Nelson 1994; Naruse 1996). The additional duplications of *Hox* clusters seem to have happened at least before the divergence of Medaka and zebrafish, suggesting the ancestor of these species is probably a common ancestor of all teleosts. If this is the case, almost all teleosts should have the extra "fish" *Hox* clusters, and the lineage-specific loss of *Hox* genes or clusters, as observed in pufferfish, might explain the remarkable variation in teleost morphology.

Conservation of synteny: The *Bf/C2*, *Msx4*, and *Fgfr4* genes were linked in both Medaka and zebrafish, although the gene order was different: *Fgfr4-Bf/C2-Msx4* in Medaka and *Bf/C2-Fgfr4-Msxd* in zebrafish (Postlethwait *et al.* 1998). These results suggest a rearrange-

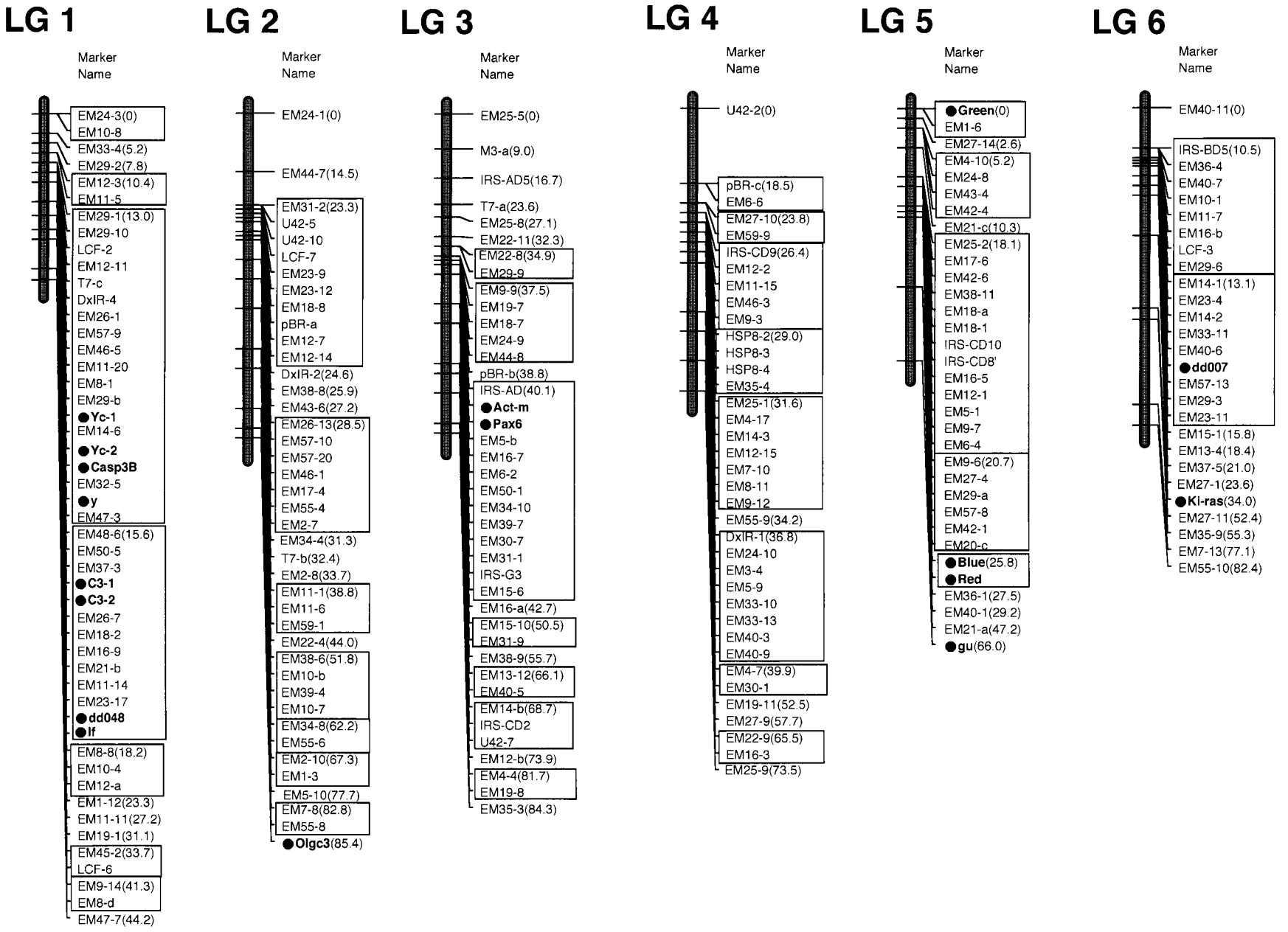

Figure 1.—A genetic linkage map of Medaka *Oryzias latipes*. Map distance from the top locus is described in terms of centimorgans (Kosambi mapping function) in parentheses. The EST and phenotypic markers are described with boldface characters. Markers in the box showed no recombination in 39 meioses.

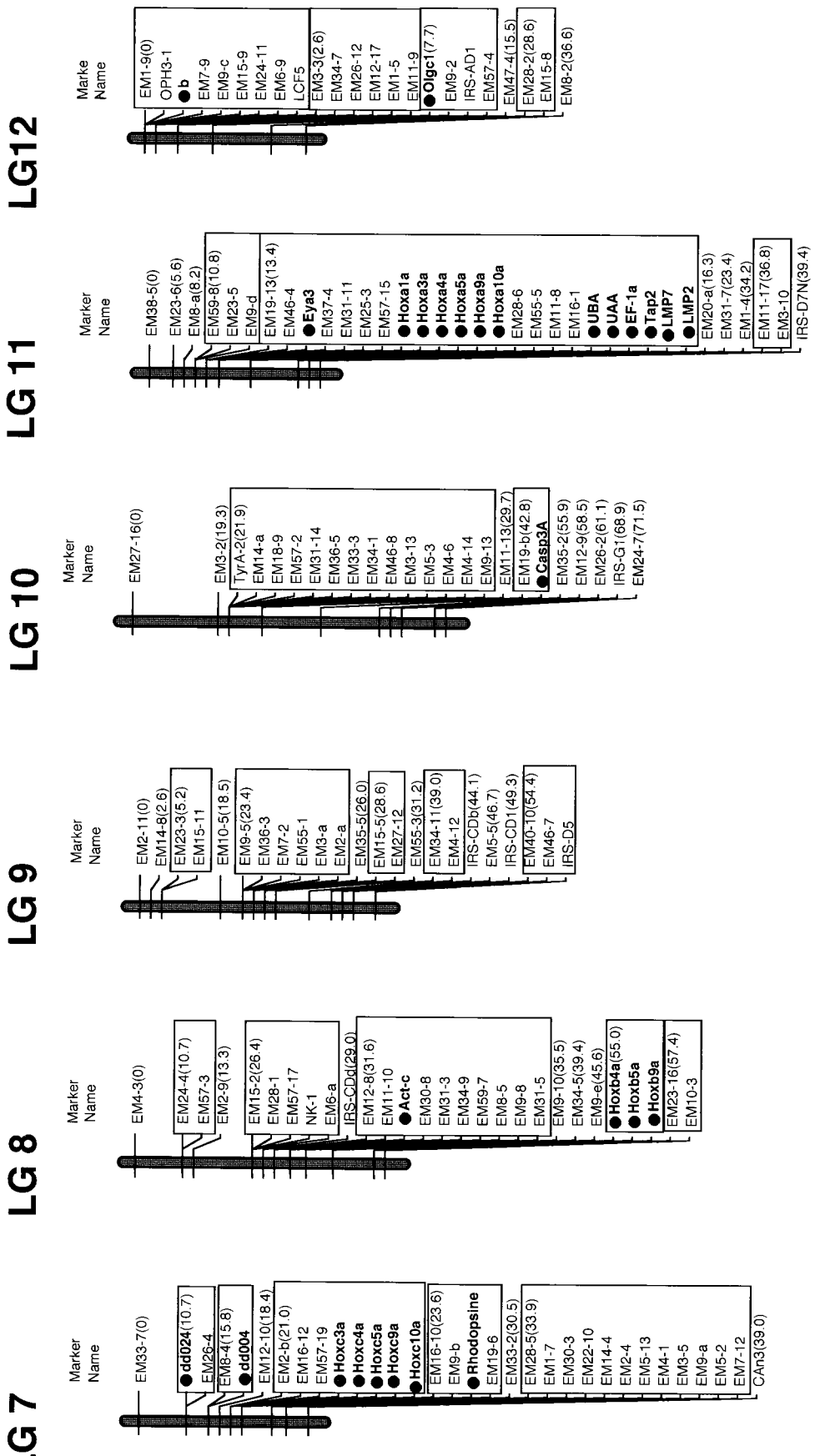

Figure 1.—*Continued.*

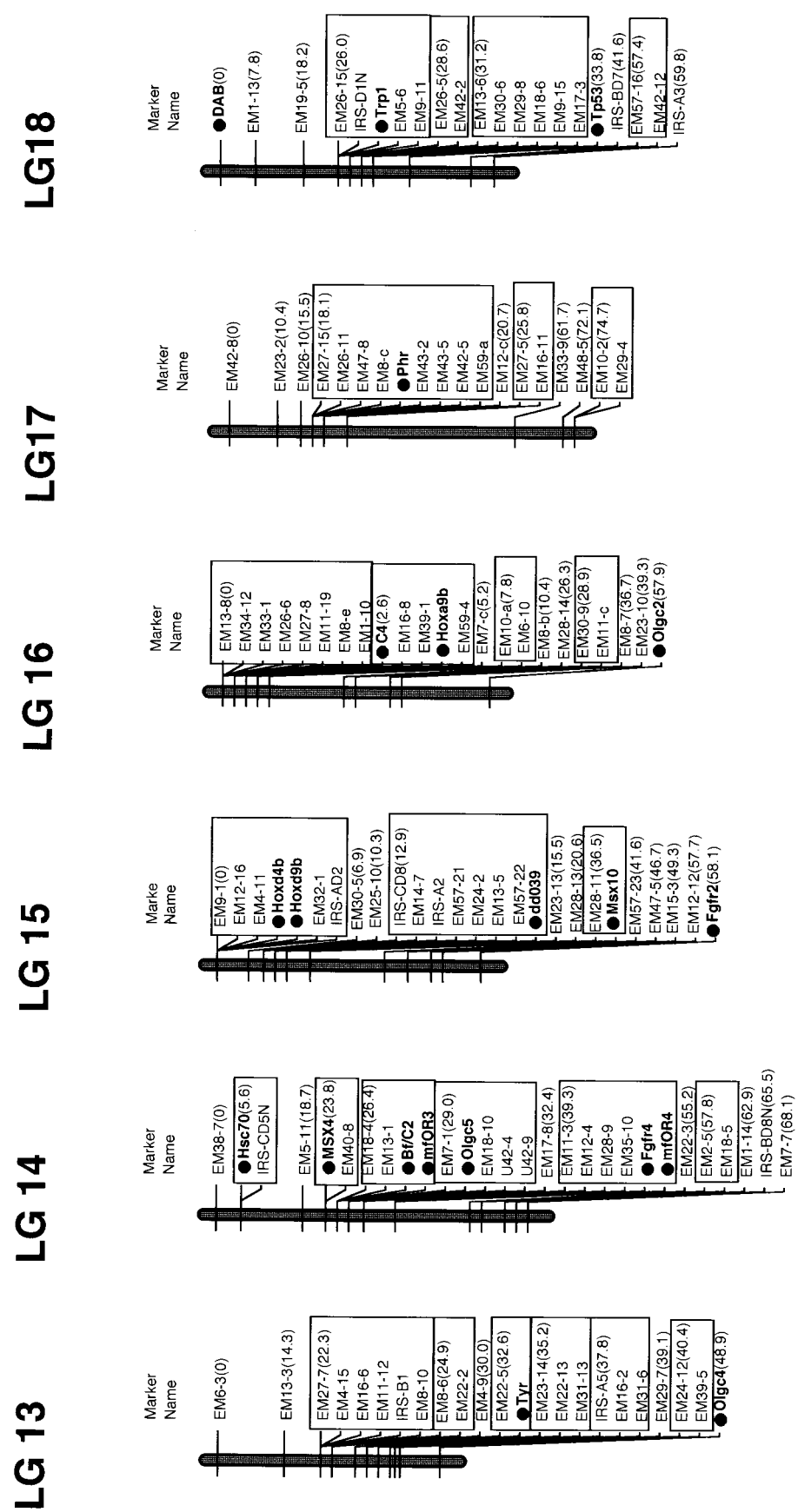

Figure 1.—*Continued.*

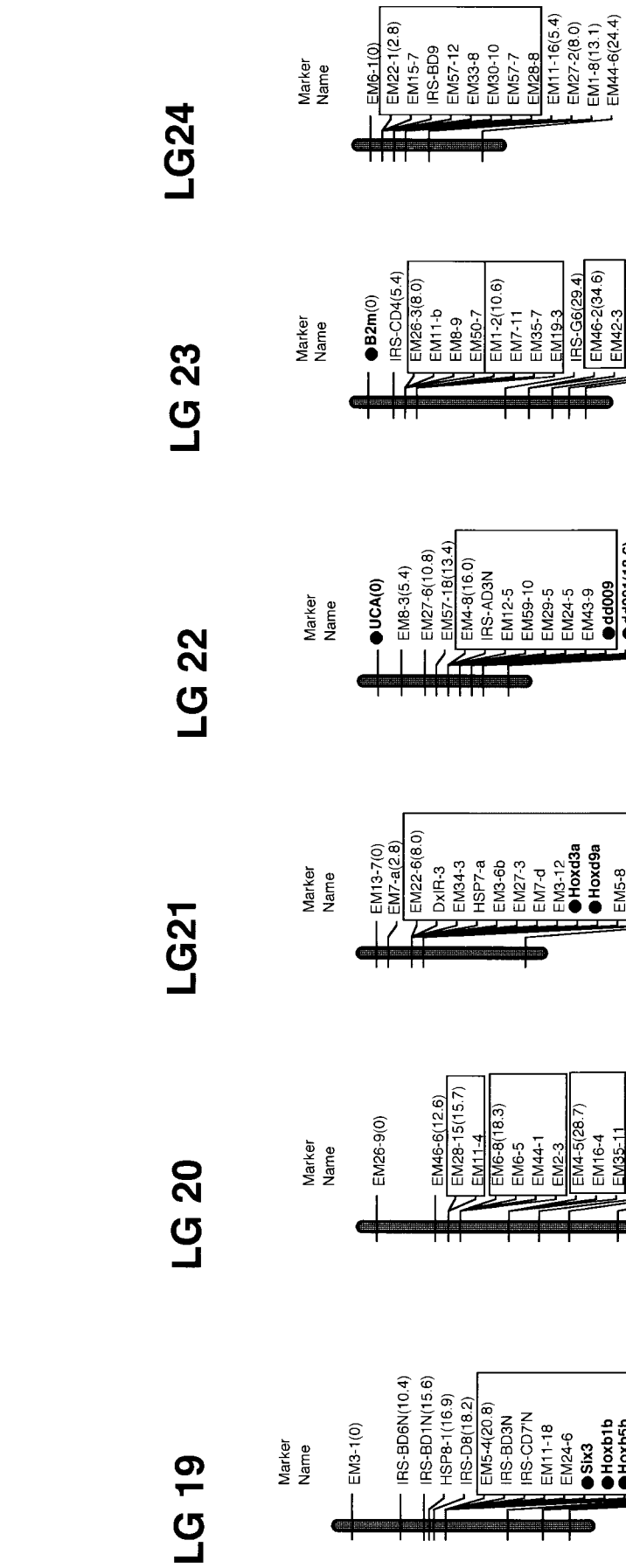

Figure 1.—*Continued.*

TABLE 4

Distribution of markers and length for each linkage group

LG	Length (cM)	Total no. loci (A)	No. of ESTs & phenotypic loci (B)	No. of anonymous DNA loci (A-B)	Expected no. of anonymous DNA loci (D)	(A-B)/D
1	44.2	49	8	41	18.08	2.27
2	85.4	41	1	40	34.93	1.15
3	84.3	40	2	38	34.48	1.10
4	73.5	37	0	37	30.06	1.23
5	66.0	33	4	29	26.99	1.07
6	82.4	27	2	25	33.70	0.74
7	39.0	32	8	24	15.95	1.50
8	57.4	28	4	24	23.48	1.02
9	54.3	23	0	23	22.21	1.04
10	71.5	24	1	23	29.24	0.79
11	39.4	35	13	22	16.11	1.37
12	36.6	23	2	21	14.97	1.40
13	48.9	23	2	21	20.00	1.05
14	68.1	28	7	21	27.85	0.75
15	58.1	26	5	21	23.76	0.88
16	57.9	23	3	20	23.68	0.84
17	74.7	19	1	18	30.55	0.59
18	59.8	21	3	18	24.46	0.74
19	47.4	21	4	17	19.39	0.88
20	71.8	17	1	16	29.37	0.54
21	32.3	16	2	14	13.21	1.06
22	29.7	18	4	14	12.15	1.15
23	47.4	16	2	14	19.39	0.72
24	24.4	13	0	13	9.98	1.30
Total	1354.5	633	79	554	554	

ment in gene order between the two fish. The synteny among MHC class I A genes, *LMP2*, *LMP7*, *Tap2*, and the *HoxA* cluster loci was conserved between Medaka (LG11) and zebrafish (LG19; Bingulac-Popovic *et al.* 1997; Postlethwait *et al.* 1998). The *Ef-1a* locus was located on LG11 in Medaka. In mammals, the MHC class I A genes, *LMP2*, *LMP7*, *HoxA* cluster, and *Ef-1a* loci, were mapped to the same chromosome (http://www4.ncbi.nlm.nih.gov/Omim/). Thus, conserved synteny was found between the mammals, zebrafish (Amores *et al.* 1998; Postlethwait *et al.* 1998), and Medaka. Further accumulation of mapping information for orthologous genes of Medaka and other fish would promote an understanding of genome evolution in vertebrates.

Sex chromosomes and sex-linked genes: Although the Medaka sex chromosome was cytologically identified as one of the largest chromosomes by fluorescent *in situ* hybridization (FISH) analysis, no difference in morphology between the *X* and *Y* chromosomes was observed (Matsuda *et al.* 1998). If the size of the chromosome and recombination frequency represent the physical size of the DNA in the chromosome, the largest number of markers should be detected on LG1. Although LG1 has the largest number of markers (49), map length was 44.2 cM, which corresponds to only ~20 markers (633 × 44.2/1354.5; see Table 4). On LG1, two large clusterings of markers were observed, one with 19 markers and another with 13 markers. Map distance between these two regions was 2.6 cM, which corresponds to only a single crossover event in the 39 fish. Such large clusters of loci were not observed in zebrafish chromosomes, strongly suggesting a restriction of recombination on Medaka's sex chromosomes compared to its autosomes, although no morphological differences between *X* and *Y* chromosomes were observed.

The map presented in this study is the most detailed linkage map of Medaka currently available and has the marker density equivalent of a skeletal level map. Using the reference typing panel established in the present study, we can easily map newly identified genes and DNA markers linked with phenotypic traits of interest, without misassignment of genes or ambiguities in gene order.

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