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A reference cross DNA panel for zebrafish (*Danio rerio*) anchored with simple sequence length polymorphisms

Ela W. Knapik*, Alec Goodman, O. Scott Atkinson, Carole T. Roberts, Masahide Shiozawa, Chäng U. Sim, Sarah Weksler-Zangen, Maria R. Trolliet, Corey Futrell, Brendan A. Innes, George Koike, Michael G. McLaughlin, Luc Pierre, Jason S. Simon, Eduardo Vilallonga, Millie Roy, Pei-Wen Chiang, Mark C. Fishman, Wolfgang Driever and Howard J. Jacob

Cardiovascular Research Center, Massachusetts General Hospital - East, 149 13th Street, Charlestown, MA 02129, USA

*Author for correspondence (e-mail: knapik@helix.mgh.harvard.edu)

SUMMARY

The ultimate informativeness of the zebrafish mutations described in this issue will rest in part on the ability to clone these genes. However, the genetic infrastructure required for the positional cloning in zebrafish is still in its infancy. Here we report a reference cross panel of DNA, consisting of 520 F_2 progeny (1040 meioses) that has been anchored to a zebrafish genetic linkage map by 102 simple sequence length polymorphisms. This reference cross DNA provides: (1) a panel of DNA from the cross that was used to construct the genetic linkage map, upon which polymorphic gene(s) and genetic markers can be mapped; (2) a fine order mapping tool, with a maximum resolution of 0.1 cM; and (3) a foundation for the development of a physical map (an ordered array of clones each containing a known portion of the genome). This reference cross DNA will serve as a resource enabling investigators to relate genes or genetic markers directly to a single genetic linkage map and avoid the problem of integrating different maps with different genetic markers, as must be currently done when using randomly amplified polymorphic DNA markers, or as has occurred with human genetic linkage maps.

Key words: zebrafish, genetic map, reference cross

INTRODUCTION

Genetics, and in particular, genetic linkage maps are helping investigators locate genes involved in essential biological processes. Linkage maps have greatly accelerated the localization of genes in agricultural crops and animals, human, mouse, rat and most recently zebrafish. As outlined by several reviews (Kimmel, 1993; Mullins and Nusslein-Volhard, 1993; Driever et al., 1994; Fishman and Stainier, 1994; Nusslein-Volhard, 1994; Kimmel et al., 1995; Kuwada, 1995; Driever, 1995) and demonstrated in this issue, the zebrafish is an extraordinary genetic model system and it will play a major role in the identification of genes involved in normal and pathological processes of vertebrate development.

However, the question now is: how are the genes responsible for these mutants going to be identified? Some of the mutants will be caused by mutations in known genes having similar effects in other organisms, but the majority of the mutated genes will be found using various positional cloning techniques (Collins, 1992). Success of the positional cloning projects will depend on: (1) a large number of genetic markers (spaced every centiMorgan) whose order is well defined, (2) several different physical mapping tools: yeast artificial chromosomes (YAC) libraries, bacterial artificial chromosomes (BAC) libraries, somatic cell hybrid panels, (3) radiation hybrid panels, and (4) a little luck.

The first step in positional cloning is mapping the phenotype of interest to as small of a genetic interval as possible, typically about 1 centiMorgan (cM) or 625 kb for zebrafish (Postlethwait et al., 1994). Since the zebrafish genome is in 25 chromosomes (Endo and Ingalls, 1968), with an estimated genetic size approx. 2635 cM (c.f. Postlethwait et al., 1994) and a physical size of 1.7×10^9 bp (Postlethwait et al., 1994), approximately 2000 genetic markers will be required. These genetic markers need to be transferrable between crosses with a high degree of fidelity. The construction of the first genetic linkage map based on randomly amplified polymorphic DNAs (RAPDs) by Postlethwait et al. (1994) and commercial availability of these markers has set the foundation for the genetic map and demonstrated the utility of a genetic map for zebrafish. RAPD markers, while necessary and useful at this early stage of zebrafish genomics, are of limited utility until the loci are cloned and primers designed to selectively amplify these loci. Currently, RAPDs are difficult to use in diploids and it is necessary to generate a complete genetic linkage map every time a different cross is made. Proliferation of unique genetic linkage maps for every cross will make it difficult to integrate the various maps. Map integration is a challenging endeavor given that zebrafish strains are not inbred. The literature on genetic mapping in humans provides ample evidence of how difficult map integration can be, and of the amount of duplicate work it requires.

The European Backcross Collaborative Group for the mouse recently developed a panel of 982 backcross progeny that can be used for high resolution mapping, conserved order map comparisons and as the starting point for a physical map (Breen et al., 1994). Unfortunately, this cross was not the same one used to construct the genetic linkage map. Thus 1000 to 2000 genetic markers already placed on the genetic linkage map, must now be mapped on the backcross, resulting in marker substitutions (since some markers are not polymorphic in the backcross) and a tremendous duplication of effort. Even so, this first attempt to assemble a large reference panel of genetically related progeny has shown the power for placement of markers into a 'fine' order (0.1 cM).

To prevent the need for multiple genetic maps, we have used a strategy similar to that used for the mouse, and developed a DNA resource for mapping. We report a reference cross DNA panel for the zebrafish, consisting of 520 F_2 progeny (providing 1040 meioses) derived from a single set of F_1 parents and anchored to the genetic linkage map with 102 simple sequence length polymorphisms (SSLPs). The 102 SSLPs selected from the framework map are a subset of the more than 500 markers, we have now placed on the genetic linkage map. This initial set of SSLPs can now be used to map any cross (assuming the markers are polymorphic in the strains used). Since the genetic map cross is a subset of animals from the reference cross, map integration is automatic. We will provide DNA from this cross to the community, allowing for the placement of newly isolated genes and genetic markers on this cross. As genes are added to this cross, the degree of conserved gene segments with other organisms will become apparent and provide the foundation for identification of positional candidate genes.

MATERIALS AND METHODS

Cross design

The strains used for the reference cross were derived as follows: AB strain fish (Chakrabarti et al., 1983) were obtained from the University of Oregon, Eugene, zebrafish facilities and maintained at Boston through four generations of inbreeding, and selected in each generation for absence of embryonic lethal phenotypes. A fourth generation female from this line was used for the cross. India (IN) strain fish originated from a collection of wild fish from the northeast of India in 1990. These IN fish were maintained through three generations of inbreeding, and a third generation male was used for the cross. Due to limited breeding and selection, IN fish are probably not free of mutations reducing larval viability.

Among the F_1 progeny, one pair was selected to generate 790 F_2 fish. This F_1 pair was selected from ten tested pairs, since their progeny was free from developmental defects, since less than 25% of embryos or larvae died during the first 2 weeks of development. To generate the entire set of 790 F_2 progeny, 11 egg clutches were obtained from crosses at weekly intervals, a total of about 1270 fertilized eggs. The balance of 480 represents fish that died at various stages, or could not be sexed unambiguously. All 790 F_2 progeny were grown to adulthood and killed in accordance with the Massachusetts General Hospital Guidelines for Care and Use of Animals.

DNA extraction

Each fish was homogenized in 2 ml of phosphate-buffered saline for 15 seconds using a polytron (Brinkmann). 12 ml of lysis buffer (50 mM Tris-HCl (pH 8.5), 100 mM EDTA (pH 8.0), 200 mM NaCl), 1 ml of Proteinase K (10 mg/ml), and 1 ml of 20% SDS were added. Samples were mixed by inversion and incubated at 50°C overnight. DNAs were precipitated with isopropanol after an extraction step with phenol/chloroform (1:1 volume). The DNA was suspended in 3 ml of deionised H_2O and the concentration of the nucleic acids assessed using spectrophotometry. For genotyping, the DNA was diluted to 4 ng/μl.

To protect against sample cross-contamination, every DNA sample was screened with two genetic markers that have four alleles segregating. Any sample that showed more than two alleles was removed from the data set and the DNA discarded. Duplicate DNA master plates (96 wells × 5 plates × 2 for the duplicate) were made and all PCR amplifications were derived from these plates. To ensure that there were no sample mix-ups between the primary plates and the duplicate plates, five genetic markers were typed in both sets and the genotypes were compared. All five genetic markers produced the same genotypes in both plates.

Marker development

A zebrafish small insert genomic library was prepared as previously described (Jacob et al., 1991, 1995) with slight modifications. This library was prepared after digesting the genomic DNA from AB fish with one of the three restriction enzymes *Alu*I, *Hae*III, or *Rsa*I (New England Biolabs). The digested DNA were ligated to *Bst*XI adapters (Invitrogen) and cloned into an M13mp19 vector (Boehringer Mannheim) modified to include two *Bst*XI sites to avoid formation of chimeric inserts and self ligation. Oligos $(CA)_{15}$ and $(GT)_{15}$ were used as probes, end-labeled with [γ^{32}P]ATP (6000 Ci/mmol, Du Pont-New England Nuclear). The genomic library (approx. 250 plaques/150 mm Petri dish) was screened by plaque hybridization using Colony/Plaque Screen (Du Pont-New England Nuclear). Hybridization was carried out at 65°C in Church's hybridization solution (Church and Gilbert, 1984) with the radiolabeled probes described above. Filters were washed at 65°C in 0.1×SSC (15 mM NaCl and 1.5 mM sodium citrate) and 0.1% SDS, and positive clones isolated.

Phage DNA was prepared using Qiagen columns (Qiagen). DNA sequencing was performed with an ABI 373A DNA Sequencer (Applied Biosystems) using the manufacturer's Taq cycle sequencing protocol.

DNA sequences containing a simple sequence repeat (SSR) were analyzed with the computer program PRIMER version 0.5 (Lincoln et al., personal communication) using the same criteria as previously described (Jacob et al., 1991, 1995). PCR primers were synthesized commercially (Research Genetics).

Selection of genetics markers

The 102 genetic markers genotyped on the DNA panel of the reference cross were selected based on the genetic linkage map (>500 genetic markers) for the zebrafish that we are constructing in our laboratory. The initial map was constructed using MAPMAKER (Lander et al., 1987). Linkage groups were determined using a two-point analysis. Local order was established by multipoint analysis. However, we have not yet employed the version of MAPMAKER designed to handle the three and four allele systems. Details of the construction of the genetic map will be published elsewhere (E. W. K., A. G., M. C. F., W. D., H. J. J. and others). For the map presented here, we selected markers from over 500 genetic markers on our SSLP map as close to 20 cM intervals as possible. This level of resolution provides investigators with (1) a starting point to use the markers immediately, while the genetic map is being completed, and (2) the opportunity to add genetic markers and genes to the map, thereby determining the genetic location relative to other genes and markers.

Allele characterization

In a non-inbred organism the number of alleles identified is determined by the size of the general population and by the sample size studied. Since the various zebrafish strains have undergone several cycles of inbreeding and breeding within a closed colony, we selected three fish from four different strains: AB, IN, Tübingen, (Tü) and Top

Long-fin (TL). Allele sizes were then determined by genotyping each of the 102 markers on all 12 fish, and by comparing the amplified product sizes to a known size standard (pBR322 digested with MspI, New England Biolabs). The G_0s of the AB × IN reference cross were characterized separately.

Genotyping

The progeny of the AB × IN F_2 intercross was genotyped as previously described (Jacob et al., 1991, 1995) with a modification to the PCR protocol: initial denaturation at 94°C for 3 minutes, 27 cycles of 92°C for 1 minute, 58°C for 1 minute and 72°C for 1.5 minutes, and a final extension period at 72°C for 7 minutes.

Map construction for the reference cross

Linkage analysis was performed using the MAPMAKER computer package (Lander et al., 1987). Linkage groups were constructed using two-point analysis and local order was determined using multipoint analysis. This framework map was then compared to the genetic map. The command 'ripple' was used to check whether the order of the markers in the framework map were correct.

To minimize the number of errors, we utilized the automatic error detection package in MAPMAKER (Lincoln and Lander, 1992), which flags double crossovers. Potential errors were checked against the autoradiograms and, where necessary, genotypes were repeated. All films were read at least twice and data entry was checked for mistakes.

As part of a project to integrate the RAPD map with the SSLP map, we have been working with Drs. John Postlethwait, Will Talbot and Michael Gates. While these data will be presented elsewhere, when applicable (Fig. 3).

Nomenclature

All loci are SSLPs and are named in accordance with lab rules, where Z denotes the zebrafish and the number indicates the clone selected during the hybridization screen. It is important to bear in mind that these numbers are assay names only. Formally, once the map is completed and physically linked to each chromosome, each SSLP should receive a locus name. We suggest a system similar to that used in the dog, mouse, pig, and rat. The name consists of a species designation DR – for *Danio rerio*, followed by a D (for DNA marker), chromosome number, institution name (or lab name) and number based on the sequential placement of new markers for that chromosome by that group, e.g. *DR-D1Mgh1* for the first Mgh marker on zebrafish chromosome 1. Where no confusion can result, the prefix *DR–* can be dropped. Gene names should follow the nomenclature rules for the human.

RESULTS

Strategy for constructing the reference cross

We have generated a collection of 790 F_2 progeny from a single set of F_1 parents derived from a G_0 cross (AB × IN). The use of a single set of F_1 parents removes the complications associated with generating maps in non-inbred organisms; therefore, a maximum of four alleles could segregate at any given marker (Fig. 1).

Our goal was a minimum of 500 F_2 fish (1000 meioses) genotyped for each genetic marker; therefore, we selected 520 DNA samples. The 102 markers genotyped were selected from the mapping panel, which contains 44 F_2s from the reference cross (Fig. 2). On average, we lost 20 genotypes per locus; yielding approx. 500 genotypes per locus (96% success rate). This translates to an equivalent of 1000 meioses with a maximum mapping resolution of 0.10 cM (100cM/1000 meioses). On a per fish basis this means that on average 3.8 loci were not typed (3.9%) in any given animal, with one notable exception. Sample 357 failed to produce a genotype in 99 out of 102 loci.

Although our stocks of DNA for F_2s are not infinite, we obtained on average 0.5 mg of DNA per sample. Since the PCR protocol used requires a maximum of 10 ng of DNA per reaction, we are able to extend its use to at least 50,000 PCR amplifications.

Allele characterization

Allele sizes of all 102 SSLPs were determined in three fish from four commonly used strains: AB, IN, Tü, TL, plus the G_0s, as shown in Table 1. These allele sizes are relative and should only serve as a guide, since we did not use an internal size marker in each lane. The predicted size is based on the sequencing of the clone containing the SSR. The predicted size and primer sequences are shown in Table 2. In 11% of the cases the size of the amplified fragment denotes a new allele. It is important to note that while a large proportion of the markers are polymorphic between the four strains, there are also a large number of shared alleles amongst strains, about 30%. The degree to which the markers with shared alleles are informative depends upon the chance segregation of the common allele occurring in both G_0s and the shared alleles being passed to both F_1s.

As shown in Table 1, there are a large number of shared alleles between the different strains and the G_0s. We chose not to use markers with shared alleles in the F_1s (data not shown), thereby maximizing the map information content of the markers.

Genetic map

The location of 97 genetic markers on the framework map is shown in Fig. 3. Five genetic markers remain unlinked. Collectively the map and the unlinked markers cover 2520 cM (using the Kosambi map function). The markers on the map

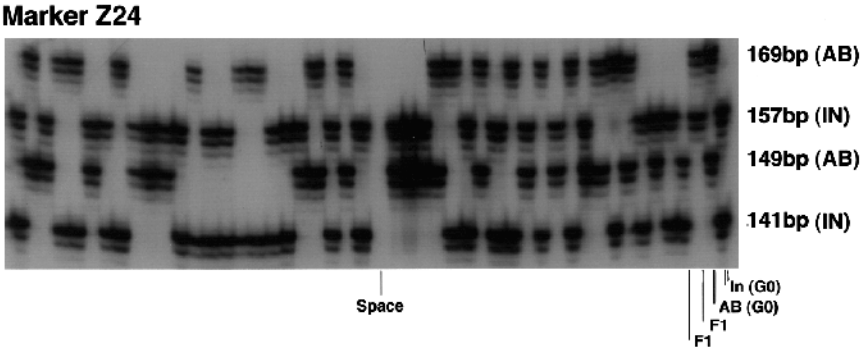

Fig. 1. Genotypes of F_2 progeny for the mapping cross for marker Z24. Four alleles are shown, two for each strain. Segregation of these alleles can be seen in the F_2 progeny at the predicted Mendelian ratios of 1:2:1 for homozygous for the AB alleles, heterozygous for the AB and IN alleles and homozygous for the IN alleles. The characteristic slippage seen for CA repeats is evident.

Table 1. Allele sizes of simple sequence length polymorphisms in 4 strains

Assay name	Predicted size	AB			AB	IN	IN			TL			Tü		
		1	2	3	G_0	G_0	1	2	3	1	2	3	1	2	3
Z7	145	145/147	145/147	147	147	143	147	143	139	145	137/147	145	147	147	139/143
Z24	149	169	149	169	149/169	141/157	139/157	187	141/187	141/149	145/149	145/149	137	137	137
Z249	147	147	147/149	147/149	147/149	141/151	139/141	141/151	141/151	149	147/149	147/159	145/147	147	145/147
Z266	212	202/212	202/212	202	202/212	192	192	192	194	202	202	202	200	200	200
Z342	163	163/169	163/169	163	169	155/189	–	155/235	155/235	235	235	235	163/235	235	–
Z374	166	166	166	166	166	194/206	194/206	194/206	206	172	166/172	166	166	166	166
Z379	222	222/260	222/260	222/260	260	252	–	228/252	–	–	228/238	–	232	232	232
Z450	154	148	148	142/154	148	132/170	132	142/170	142/170	148	148	148/154	142	142	142
Z470	139	113	139	113/139	113/97	99	117	117	117	–	–	–	139	139	139
Z536	118	118	118	118	118	124	122/124	124	120/124	122	110	122	110	110	110
Z562	207	207	197	207	207	199	199	199	199	199/203	199/203	199/203	203	203	203
Z644	144	144	144	144	144	140/146	146	140/146	140/146	148/150	148/150	146/148	146	146/148	146
Z728	204	204	204	204	204	201	206	206	206	210	204/210	204	204	210	210
Z732	133	–	103/133	133	133	135/155	121/155	117/155	129	103/141	99/103	99/103	103	103	103
Z737	126	100	108	108	108	98	98	98	–	–	–	–	108/112	112	112
Z787	111	–	111	111	125	111	111	111/131	111/131	109	111/125	111	111	111	111
Z789	203	203	201203	201	201	203	203	199/203	199/203	191/201	191/201	191/201	203	203	203
Z868	128	128	128	128	128	114	118	114	114/118	112/128	128	112/116	128	128	128
Z872	220	220/236	236	220/236	236	228	–	228	228	220/232	220/232	220	304	220	220/304
Z880	150	160	150/160	150	150/160	170	126/160	170	126/170	112/160	142/154	126/148	142	142	142
Z928	134	134	134	134	94	106	154	154	154	–	–	–	126	126	126/134
Z953	98	98	98	98	98	100	100	100	100	98	100	100	100	100	100
Z963	201	201	201	201	201	189/277	–	189/197	201	187	187/201	187/277	187	187	187
Z1059	150	150	150	150	150	124	124	124	124	–	130	–	122/150	122	122/150
Z1068	132	114/122	122/132	122/132	122	132	132	114/122	114/122	122	122/132	122/132	114/122	114/122	114/122
Z1148	200	200	200	200	200	238	238	196/238	196/238	238	174	–	174	174	174
Z1162	134	134/136	134/136	134/136	134	134/140	134/136	134	134	–	134	134/140	134	134	134
Z1197	225	175/189	175/189	225	225	229	–	203/229	203/249	189	189	221	189/207	189	189/207
Z1209	150	140/160	140/150	140/150	140	128	140	128/140	128	150	134/150	150	140/150	140/150	150
Z1213	127	127	127	127	127	117	117	167	141/167	127	109/127	127/139	127	127	127
Z1239	190	197/221	197/221	197	197	237/269	–	237/269	269	175	175	175	175/263	175/263	175
Z1243	191	191	191	191	191	201	201	201/309	201	–	217/239	107	243	243	243
Z1257	123	117/123	117/123	117/123	123	117	117	109/117	117/119	117	109/117	117/123	117	117	117/123
Z1265	140	102/140	102/140	102/140	102/140	114	128/138	114	138	102/106	102/140	102	102/106	102/106	102
Z1276	279	267/279	267/275	267	279/293	275	–	275	275/309	–	267	–	267/293	267/293	267
Z1296	258	250/258	250/258	258	258	250	242	250	250	–	258	258	258	258	258
Z1312	139	139/181	139/181	139/181	139/181	139	105/107	105/181	105/181	111	139	111	181	181	181
Z1351	136	138	136/138	136	138	164/222	164	164	164	138	138	138	222	222	164
Z1366	119	119/121	119/121	119	119/121	103/111	111/121	103/121	121	121	111/121	111	111/139	111	97/111
Z1368	103	99	99	99	99	103/97	103	103	103	–	–	–	101	99/101	101
Z1396	150	153	153	153	153	141	141	141	141	141	141	141	141	141	141
Z1408	101	101	–	101	101	280	280	280	266/280	101	101	101	101	101	101
Z1411	144	144	144	–	144	112	–	–	–	–	–	–	112/132	112/132	132
Z1416	136	130/136	130/136	130/136	130	126	126	126	126	130/136	126/130	126	134	134/136	134
Z1431	149	137/149	137/149	149	137	149	147	169	169	–	137	149	137	137	137
Z1473	149	196/210	196/210	196/210	196/210	192	–	192	–	–	–	–	190	–	190
Z1490	135	135/137	135	135	137	147	139/147	147/179	147/179	121/147	139	139	135	135	135
Z1492	246	246	246	246	246	214	214	214	214	–	244	–	218	218	218
Z1536	199	199	199	199	199	195/213	199	195	199/201	–	199	–	213	213	213
Z1625	211	199	199	211	211	167	–	167	213	143	143/211	143	213	211	211/213
Z1628	186	146/186	146/186	146/186	186	166	132/166	132/146	132/146	–	186	138	190	190	190
Z1637	105	105/143	105/143	105/143	105/143	103/109	109	101	101	105	107	107	133	133	103/133
Z1796	228	228	228	228	228	230	228	228	228/230	–	228	–	228	228	228
Z1803	183	183/189	183/189	183/189	189	175/183	183	175/183	175/183	–	195	–	175	175	175
Z1810	160	110	110	110	110	122	–	122	–	110	110	110	110	110	110
Z1842	196	–	196	196	196	198	–	198	198	–	180	–	210	210	210
Z3077	151	139	135/139	135/139	139	135	–	135/139	135/139	–	139	–	139	–	139
Z3093	136	136/146	146	146	136/146	164	–	164	164	164	146/164	146/164	146	136/146	146

Table 1. Continued

Assay name	Predicted size	AB 1	AB 2	AB 3	AB G_0	IN G_0	IN 1	IN 2	IN 3	TL 1	TL 2	TL 3	Tü 1	Tü 2	Tü 3
Z3104	254	339	259/349	339	339	283	–	247	283	–	251	–	269/309	269/309	269/309
Z3124	127	135	135	135	135	131	131	131/133	131/133	133/135	133/135	133	213	141/213	141/2
Z3157	127	113/127	113/127	113	113/127	109	109	109	109	109	109	109	109	109	109
Z3286	100	94	94	94/100	94/100	94/98	–	94	94/98	94	94	–	–	94	–
Z3310	150	142/150	104/142	104/142	104	144/150	196	144	144	144	144	142/148	234	150	234
Z3490	141	–	141/165	141	141	165	153	129/153	129/153	141	153/165	153/165	141/165	165	141
Z3527	226	226/286	226/286	286	286	220	220	220	220	286	202/286	202	202	202	202
Z3602	107	105/107	105	107	105/107	87/129	–	87/107	87/129	–	107	–	123	123	123
Z3725	256	248/262	248/262	256	248/262	296	–	248	248	–	256	242	248	248	248
Z3745	141	121/141	121/141	121/141	121/141	137	121	121	137	141	141	–	145	145	145
Z3763A	–	260/266	260/274	266/274	266	260	260/266	260/266	266	–	–	–	260	260	260
Z3763B	–	180/190	190	180/190	190	180	–	180	180	180	180/190	180	180	180	180
Z3763C	147	147/159	147/159	147	147	159	159	159	159	147/159	147	147/159	147/159	147/159	147/159
Z3782	115	93/115	115/201	115/201	93/115	143/201	–	115/143	201	115	163	115	115/201	161	115/161
Z3835	153	120/134	120	134	122/132	230	132	132	132	–	–	–	120	120/142	120
Z4003	225	229/247	225/247	225/247	225/247	197	197	197/269	197/269	197	197/239	197	197	197	197
Z4009	281	275/281	275/281	249/281	281	233	241/243	233/241	243/275	–	289	–	289	249/289	289
Z4120	145	139/145	139/145	139/145	139/145	141/143	141	133/143	141/143	143/145	143/145	133	139/143	143	143
Z4188	207	207	207	207	207	203	203	227	227	–	197/207	–	217/233	217/233	217/233
Z4263	314	314	314	314	324	318/320	318	318	318	–	–	–	–	314	314
Z4291	147	147	147	147	147	135/159	141	135/159	129/153	129	157/169	147	143/153	141/143	143/153
Z4299	198	262/308	262/308	262	308	202/206	202	202/206	202/206	202/206	202	–	228	228	228
Z4332	175	165/175	175	175	175	165	165	165	165	173	173	–	173	173	173
Z4507	239	231/239	217/239	231	231	223	–	223	223	223	231	–	231	231	231
Z4670	147	147	139/147	139	139	137	135	127/131	131/137	143	161	147/161	147	147	147
Z4706	188	188/200	188/200	188	188	200	–	188/200	200	–	–	–	196	196	196
Z4830	194	194	162/194	194	162	176/288	188	204/288	204/288	162/194	194/220	194/198	176/198	198	176/198
Z4862	177	177	177	177	177	173	173/175	175	175	175	173/175	175	173	173	173
Z4910	362	414	398	414	398	414	–	398/414	398/414	–	–	–	414	414	414
Z4951	251	251/257	251/257	257	257	245/273	273	273	245/256	–	257	–	245/251	245/251	257
Z4956	263	–	239	239/263	263	239	239/263	263	239/263	239/263	–	239/263	239/263	239/263	239/263
Z5033	253	261	253	253	253	277/361	–	239/277	277/361	–	–	–	309	309	309
Z5058	185	185/191	185/191	185/191	185	177/195	195/203	177/183	177	191	191/213	191/213	185/191	185/191	191
Z5075	234	234	216/234	216	216/234	198/222	198/222	234	222/234	–	198/234	–	198	198	198
Z5081	211	211	211	211	211	189	211	189	189	–	211	–	–	–	–
Z5183	143	143	143/147	143	147	143	141/143	143	143	143	143	143	147	147	147
Z5208	347	341/351	341/351	347/351	341/351	347/359	-	347/351	359	–	–	–	347/351	347/351	–
Z5223	150	150/178	150/178	150	150/178	198/230	188	198	198/230	–	178	–	150	150	150
Z5260	261	232	232	232	232	230	230	230/232	230/232	230/232	230	230/232	230	230	230
Z5294	343	343/363	343	343	343/363	351/369	351/369	351/369	351/369	–	–	–	313/343	313/157	313
Z5352	149	149	145/149	145/149	149	147/151	147	147	147	145/151	145	151	147	147	147
Z5424	233	360/364	360/364	360/364	364	360	–	360	360	–	–	–	360/364	360	–
Z5564	236	236	236/249	236	236/249	229/239	253	229/253	229/239	–	249	–	236	236/253	253
Z5669	229	–	–	229/245	215/229	217/233	–	233	233/247	–	229	–	251	251	251

The assay name is the clone number. The predicted size is based on the length of PCR product predicted from the sequenced clone. AB, IN, TL and Tü are the strain designations. Numbers 1,2,3 under each strain of zebrafish designate an individual fish. The G_0's were the orginal strains used for the cross. The / denotes the two alleles at the given locus. – denotes a missing allele or failed PCR amplification.

were chosen to provide a comprehensive representation of the known linkage groups in the over 500 SSLP genetic linkage map. However, this genetic linkage map has not been completed to the point of attaching the markers physically to the chromosomes. This limitation prevents us from knowing, with complete certainty, whether each linkage group represents one of the 25 chromosomes. Therefore, the map presented here must be considered to be an approximate framework map.

Coverage of the genome, in cM, can be estimated from the sum of the intervals plus the sum of coverage provided by the genetic markers at the ends of each linkage group and by estimating the coverage of the five unlinked makers (typically approx. 20 cM on either side). The coverage between the intervals totalled 1320 cM and the coverage at the ends of each linkage group (2×25 linkage groups $\times$ 20 cM) totalled 1000 cM, plus 200 cM ($5 \times 2 \times 20$ cM on either side) covered by

Table 2. Primer sequences flanking the simple sequence repeats

Assay name	Left primer (5′)	Right primer (3′)	Predicted size (nt)
Z7	TCATGAGCAGCGTTTTTCC	AAAGCACCATCCCACAAAAC	145
Z24	CACCTTCACGGTGAGTAGCA	GTGGAATGGTGTGACTAATGTCA	149
Z249	TCTTCCCCTACAGGCACAGT	ATGATACGCAGTCAACGTATCG	147
Z266	TCCGGCTGACATGAGGAAT	ACGCTCTGCTTCATGGTCTT	212
Z342	CATTGAGTTTAGACCCCCACA	TCACTTCTGCTTTATAAGGCACC	163
Z374	TTTTAATCCAGCAGCAGATCTG	CCCAGGTTCACAAACCCTC	166
Z379	AGCCTTTCACTGGCCCTATT	CAAGGTGGAGTAAAAATCCTGC	222
Z450	AAGTACAGCATTGTCCACAGTCA	CAGGGAAACTGCTGATGTTG	154
Z470	GAGAGCCGAGGTGTGTATGC	CACTGCCTATTACACCACTGATG	139
Z536	ATCCAGAGTGTGTCCCAGTTG	AGGTGTGTCCCACTGACTCC	118
Z562	TGTACTTCTCTCTCAATACACCGG	AGGCCTCTGATTAACTGAATTACA	207
Z644	CACGGACAATCCAGTGTACG	GCAACAGTATAAGACTGTCTTGGG	144
Z728	GTGGTCATGTGACACAGGATG	GCTCTTACAGTCCTGAGAACAGC	204
Z732	GTGCCTTCCGTTACCTTCAC	TTTTAAGGAAAAAAAGTGAATGCA	133
Z737	AACACTTGATATTGCATGACGG	ATGTTGGTTAGCATGCACCA	126
Z787	GCTGCGTGATATTCATGGG	CTGTATCCTGCTATACAGCCCC	111
Z789	CTCCTGCTGGCCCAGTAAT	ACTGAACATTCAGGCTTAAATGC	203
Z868	GATCTCCCTAATTATAGCACTCGC	TCATCGCATCTGCAGTCAG	128
Z872	CTTAAAGCCCAGTGAGGCAC	ATGCCTGGTAATGAGCAGCT	220
Z880	TGATCTGAGACGTCTGCCG	GCGCTCTGACAGTGCTTACA	150
Z928	CCAGAGTACTCGATCTGTCTTGA	ACACTTTCGGCAGTTTTCAC	134
Z953	AGAAAAGAGTGAGGACTGTGTGC	TGCGAGTTGACAGCCTGTAC	98
Z963	ACTGGCACTTCACGCTCC	TCTCACACTGAGTGCTAAACGG	201
Z1059	AACAGGTGACAGAGCACACG	GGGAGAGGGCAGGACAATAT	150
Z1068	GGTGAGAAACACAAGAGCAGC	CAGACCCAGATTCTTCAGCC	132
Z1148	GGCTACACCTCAAACGCTTC	CAACACCTGATCAAACGCC	200
Z1162	TCTCACGCACAAACAAGGAG	TCTATTCCTGTTCTCCATACTGTCC	134
Z1197	AGTGTGTAGTCCTGCTGCGGG	ATCCTAGCTGGTAAGGCAGGGC	225
Z1209	CCTGAGCACATGTATGCAGTCG	CACAATCGCAGAGCAGCGCC	150
Z1213	CAGAAATGATGAAGCGTAAATAATG	ACCACCTCGCCCAGAGTAC	127
Z1239	AGGAAAGGCCCTGCCTTATG	TGGGCTGTACTCATTTATGTTG	190
Z1243	CGTCAAGAGAGGACGACTAACC	AAACAGATTTATACCAAGTTTACCTGG	191
Z1257	AAAAATGTCAGTCTTTCATATGCG	GAGTCCTCTCAGGCAGCG	123
Z1265	ATATGTGCTGCTCATGATGAGT	AACAGACGAAGGGTGAAGGA	140
Z1276	GATCAACCACACTGGCTGTA	GTCCTCTCCTGGAGACATCG	279
Z1296	GCCTCATTTCTCTCGTACGC	TGGGTGGGCTGTTTCAGT	258
Z1312	AGGACAACACCTGCAGCC	TGGGATGAGCTGAAGTTCCT	139
Z1351	GGTTAGTGATCAGTTAAGCAATTAGTG	CTTTCTGAAACAGGGCCAAG	136
Z1366	TACTTCAGCTTCATCCAGACCA	CTGAACTCTGAGTCCAGACCG	119
Z1368	TTCTGCATGTGCGAGCAT	AGCTGCATCTTGTGTCTAGCTG	103
Z1396	TCAGCCACTCCTTCAGTGCG	AGCGTCCTTCAGAACCTCATGG	150
Z1408	AATTCTGAAATTAAGATGTTGACCG	GAAAACGCACATCACGTGAC	101
Z1411	GCAGCTGAAACATCCAAACA	CTAAAGACATGCAGTAAATTGAGTAGA	144
Z1416	GGACACATGTTGAAACTCTACAGG	ACCTGCCCTACCCACTCC	136
Z1431	TTCTCCACATCCTGCTGAGC	GTGGTGAACGGCTCCTCTGG	149
Z1473	CAACTGGAAGGGCACGAG	ACTAAACTGGACTAGTTTGCC	149
Z1490	TGGTGTCCATGTAAACGTGCTC	GCCAGCGCAACATCTCTGAA	135
Z1492	GAGCAGCCACTCAGGTGAG	AGATACTGCTGGAGGGCTCA	246
Z1536	TCATATCTTTATCTCAGAGATGTGCG	GCACACATCTGGGGAAAACT	199
Z1625	TGGGATACCTGACATCCAGACC	GCTTGAGTGAGGACGCGGTT	211
Z1628	CAGTATCAGCACACGTGCATGC	CCGTATGAGGATGTCCACGC	186
Z1637	GGCTTTTGGATGAAGGTTGAGC	GGAATCACAATGGCAGCAGA	105
Z1796	AGACACGGGACAGCTGGGTC	TAGTTGGCGGTTCATTCCGC	228
Z1803	CAGAACCCTGCTGTTCCAGAA	AGGTGCACCGTCTGGATCTT	183
Z1810	TGACTTCCCCAAAATATTGGCC	GGAAAGGTGGCGTCAATACTGG	160
Z1842	AGCACCCGGAGGAAACCAAT	AGCCAAACTCTCCCAACAGCC	196
Z3077	TTAGCATAAGCAGCAGCCCTG	AGCGTACAGCCGGGGTTAGA	151
Z3093	GTCAGTTGGTGTCCCCCCAC	CAGCACAGGTATTATGCAGCGG	136
Z3104	TCCTTTGGAACAAGAGTGACCG	CACCAAGCGATATGTTTCCTTT	254
Z3124	CGGCTCCACAGGAACATGAC	GCCGATGCCATTTTCTGTCC	127
Z3157	GAAGGGGTCCTGGATTTGTTCA	GACACAAGTCATTGGGCAGGG	127
Z3286	CCACACATAGTGGAACCTGCTG	CTGGAAGTTACATGGAGCGTCA	100
Z3310	GCCCCCTTTGTTGATTCATTC	CAGCCCACTGAAACTCGCAA	150
Z3490	TCTAGTCGGTCCTGAGGCGC	GCAGGAAACATTGCATGACTCA	141
Z3527	TTCTGGTGTTTTCCAGCGGA	GGCAGTTCGTCACCTCAAAACA	226
Z3602	AGCATTAGTGCATTCTGCGCA	TTCCGATCCCAAAATTACAGCA	107
Z3725	ACTAAATCGCACTTCAGCAGCG	GGTGTCCTCACATCAGCTGCA	256
Z3745	AGAAGACGGTTCCCCATCGC	AGTAACTGACTTGCTGCCGCG	141
Z3763A	CCAATTAACGGAAGTGAACCGC	GCACTGGATCAGTCCCGCTT	147
Z3763B	same as Z3763A		N/A
Z3763C	same as Z3763A		N/A
Z3782	AATTCTGGGGGGTAATTCTGGC	AAGGGGGCTAAACCTTCAACTG	115
Z3835	TCACCATTTTCACAAATTGGCA	AAATGGAGATTCCGCTAAAGCA	153
Z4003	AGCAGGGGGATTCCTGCATT	AAGTTGGCCAATATGGTCTGCG	225
Z4009	AATGGTTGCCCCGTCCATC	TCATGCTGCAAACCCCAGTC	281

Table 2. Continued

Assay name	Left primer (5′)	Right primer (3′)	Predicted size (nt)
Z4120	ACAGCTGCTGCGTGATGAGC	TCTGTAAGGAGTGCCTCAGGGC	145
Z4188	GTAGTTGAATGCCGAATTGGCC	GAACACCTGCGCATCCACAG	207
Z4263	AAGCTTCACGCCAAAGACTGTG	ACAAGCACACCATTGCTGCA	314
Z4291	TTTGCTCCTCTGGACAGCACC	CCCCGAGCAGCATCACCTAC	147
Z4299	AGGAATGCGCTATGGGACGA	CACATCTGCCACTGAACCGG	198
Z4332	TAGGGTTGGGGTAGGGCCAT	TGGAATTGGAGAACATACCCCA	175
Z4507	TCACCCCATATCACTCCACAGC	TTCGGGATCTGCAGCGTGTA	239
Z4670	AGCGCTGCATACCAATGCAC	TGGTGGGTAGAAAATGCAGCA	147
Z4706	AGCTGGAAAGCCAGATGCTCA	CCCCATCAACAAAGCTCTTGC	188
Z4830	TGTTTCCTTTTGCCTGTATGG	TTTGGGGCAATAGGTGAAAG	194
Z4862	ATGACCCTTCTGTCGGAGCG	AGCCTGTCACCATTCACTGCC	177
Z4910	TTGAGGGCATCCGCTGTGTA	TGAGTGCTTCAGCACCCTGG	362
Z4951	TTCCTTCATGGTGGGCGAAT	CCTCTCGTTTGGAGCCCAGA	251
Z4956	GGGGAACGGAGGTCGAAATC	GTTGCGAGCTCCCAGCAAAC	263
Z5033	CACCAGGAAGAAATCGATGCG	GACGTTTACCCACCAAGAACCC	253
Z5058	CTGACGAGAACGGTGAACATGC	GCTCAGGGTTTGGAGGATGTG	185
Z5075	TGTGTTGTCTGATATGCCCACA	TGCAAAAAGCCTCTAAGTGCCG	234
Z5081	GCCCCCCTGAATTATTAGCACC	CGGTGAAGTTACGGTACCATGA	211
Z5183	ACAATCACTTTTGCTCTGGGCC	CCGCCATTGCAAACAGTCAC	143
Z5208	CCAAGCAACTTGAAATTTGCCA	TGCTGTAACCACTGAGGTTGCC	347
Z5223	AACAGAGCCGATCTGCCACC	AGCACAGCGGAGGAAATAAAGC	150
Z5260	CGGCGTGATGAGTGCAAAAC	CGGCTGTTTGTGTCAGAGTTGC	261
Z5294	AGGGCGGCGAAATTGAAATT	AGCTCTAACCCCCTCCAAGCA	343
Z5352	TTGCCCTGCTATCAAGGGTTG	CGTGCATCCAGCAACCAATC	149
Z5424	TCCCCAGAGATGAGTTGCAGC	TCGAGCCAGAAGACCAAGTGG	233
Z5669	TGTTGATCATCAGTTGCAGTGC	GCCTCTGCAAACAAGTTTCCG	229
Z5564	CAGTGCTAACCACAGCACCACC	CAGCCCCAACAATTTGTCCC	236

The assay name is a unique identifier specifying the particular primer pairs used to amplify the locus (as opposed to the locus itself). N/A denotes not available. The assay names ending with A, B or C designate primary, secondary or tertiary amplification products using the same primer pairs, where A is the largest product. The predicted size is from the sequencing of the clone containing the SSR.

the 5 unlinked markers yielding a total coverage of 2520 cM. Postlethwait et al. estimated the zebrafish genome to be approx. 2635 cM (Postlethwait et al., 1994). Consequently this SSLP map, although not complete provides excellent coverage of the genome. The orientation of the linkage groups is arbitrary. As would be expected, the maps constructed for the reference cross DNA panel were in complete agreement with the >500 SSLPs map, with one exception: linkage groups 8 and 21 were linked to one another. However, since the genetic map with a greater density of genetic markers did not attach these groups together, we ignored it. Five markers (Z374, Z536, Z644, Z1296, Z5075,) could not be linked together in the framework map, and the linkage groups to which they were assigned were not strong. We used these markers because they will eventually tie in with one of the existing linkage groups.

The framework map reported here, covering the majority of the genome, provides a set of anchor markers. These markers will provide precise integration points between the complete genetic linkage map and any marker or gene placed on the reference cross DNA panel. In addition, the reference cross DNA panel will provide investigators with a set of recombinant chromosomes that can be used for fine structure mapping.

Availability of data and DNA

The data underlying this map – including the primer sequences, allele sizes, and genotypes for each locus are available electronically from a server maintained at the Cardiovascular Research Center. The data can be obtained from http://zebrafish.mgh.harvard.edu. Primer sequences have also been deposited with Research Genetics (Huntsville, Alabama), where they are stocked in aliquots appropriate for mapping experiments.

A subset of 48 DNAs (44 F_2 animals, 2 F_1s and 2 G_0s) is available to the community for the initial placement of genes and genetic markers. Once the gene or marker has been

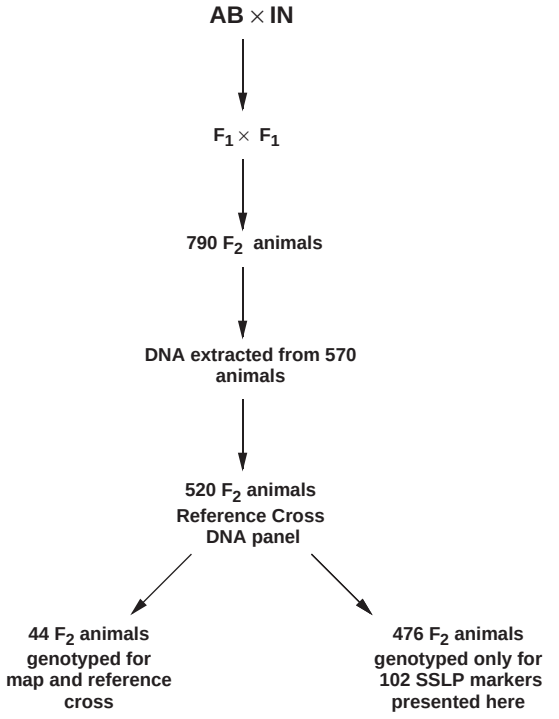

Fig. 2. A flow chart of the reference cross. The reference cross consists of 520 F_2 animals derived from the same set of F_1 parents, including the 44 used for the genetic mapping cross.

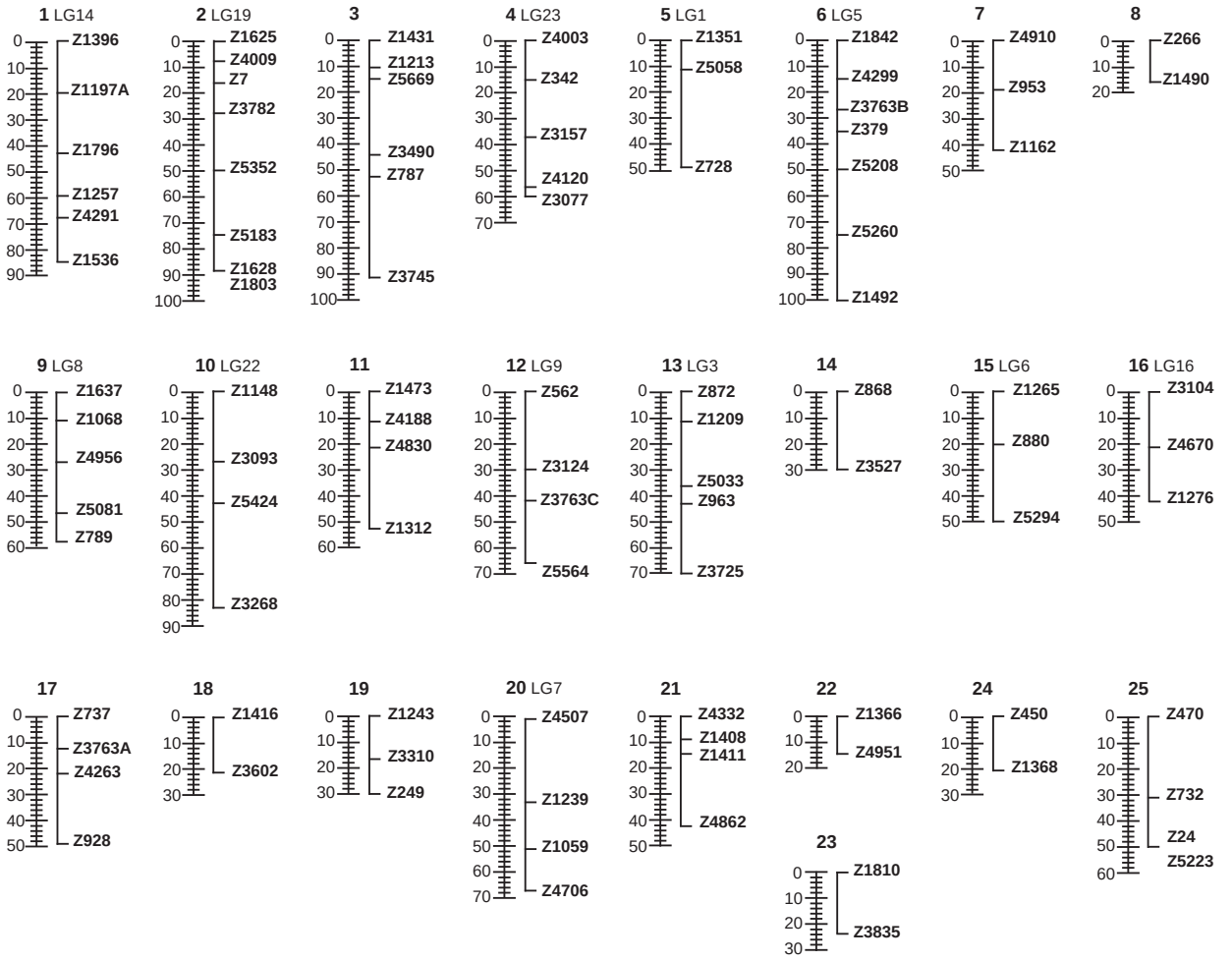

Fig. 3. The anchor map for the reference cross. The orientation is random with respect to the centromere. The distances between markers are in centiMorgans (cM), calculated using the Kosambi mapping function. Any linkage group (LG) designation is from the RAPD map. The design for this figure is from Breen et al., 1994.

mapped, DNA from the recombinant animals in the interval of interest may then be requested. Precise rules and requirements for obtaining DNA from the reference cross are also available on the zebrafish webserver at cvrc, mgh or via request. Three major requirements must be met before DNA can be released: (1) that the gene or marker be mapped using PCR, (2) that a marker has a demonstrated polymorphism between the AB and IN strains used to construct the reference cross, (3) that all mapped genes and genetic markers be posted in the reference cross database. The reference cross database will contain the actual genotypes and information regarding the gene, the primer sequences and the investigator who developed the primers. At the discretion of the investigator, the release of this information to the general public can be withheld until publication. Other guidelines, such as adherence to nomenclature rules will also need to be met.

The determination that a particular genetic marker is polymorphic is somewhat problematic given that the G_0 and F_1 DNA is relatively limited. Therefore, we will use the remaining approx. 270 F_2 zebrafish as test samples, enabling investigators to develop a polymorphic marker without having to use any of the DNA from the cross.

DISCUSSION

We have developed a genetic mapping DNA resource for the zebrafish community that will provide fine resolution mapping, prevent the need for map integration, and provide the foundation for a physical map. In addition we will provide the DNA from zebrafish known to be recombinant in any interval between the anchored markers, providing a maximum mapping resolution of 0.10 cM. Postlethwait et al. have estimated that 1 cM is approximately 625 kb cM (Postlethwait et al., 1994). Therefore, this DNA panel has the capacity to provide resolution up to 62.5 kb. At this resolution, one could contemplate making a physical map out of any large insert library, (such as a P1 library). Thus the DNA panel will provide an efficient means of obtaining a precise map position for a gene or marker and for constructing a physical map, in a region of interest.

The high resolution mapping potential and direct relationship of the reference cross DNA panel to the genetic linkage map will facilitate determination of the conserved gene segments between other organisms. The ability to order physically close genes (within 62.5 kb) will provide essential infor-

mation with regard to conserved gene sequences between the genome of the zebrafish and the genomes of other organisms.

The markers used to anchor the reference cross were selected from the genetic linkage map consisting of SSLPs that we are currently constructing. However, it is important to bear in mind three points about the map reported here. First, the genetic linkage map with SSLPs is not yet complete and none of the linkage groups have been physically assigned to a chromosome. Consequently, the linkage groups have been arbitrarily assigned as independent. Second, since the genetic map has not been completed, marker order may change as the genetic map is resolved and physically linked to the chromosomes. However, this does not detract from the opportunity to place genes and genetic markers relative to the order reported here. Since the 44 F_2 animals used to construct the genetic linkage map are a subset of the reference cross, any changes in the map can be easily corrected in the reference cross map. We will continually update the map for both the reference cross and the genetic map itself. This information will be displayed on our Web site. Third, there are likely to be errors in this data set (53,040 genotypes) and the genetic map data set. All map data sets contain some errors, even after extensive error checking. The most common error is the misassignment of a marker to the wrong linkage group. Even when a somatic cell hybrid panel is available, marker assignments have changed in the initital rat and mouse genetic maps (Howard J. Jacob unpublished data and Eric Lander personal communication). In the case of the zebrafish, linkage groups are determined by probability alone – leaving chances for more errors. The errors will be detected and corrected as additional markers are added to the map. However, the majority of the data is sound and will provide investigators with the opportunity to place genetic markers. It is important to note that the number of double recombinants is low for the approximate framework map presented here, demonstrating that the linkage groups do minimize recombinants. All recombinants were checked and in selected cases the assays were repeated to ensure that the recombinants were real. Where possible we have identified the linkage groups relative to the RAPD map (Fig. 3).

Investigators wishing to receive the DNA plate will need to contact our Web site and request a copy of the guidelines. Once the guidelines have been agreed to, the DNA plate will be sent. Due to the limited amount of DNA, only one plate can be sent to the laboratory. Investigators wishing to map a gene on the reference cross will be sent DNA from the F_2 progeny of this cross that were not used to construct the reference cross, and 44 DNAs which were genotyped for anchor markers. The investigator will develop a polymorphic marker using test DNA and upon identification of a segregating polymorphism, will genotype 44 F_2 animals as well as F_1s and G_0s from the reference cross DNA panel. The 44 animals will enable the investigator to map the gene or marker to a maximum resolution of 1.1 cM (100 cM/88 meioses). The genotype information will be incorporated into the map and into the reference cross data set. Note that the investigator will be able to use the data from the Web site to do the analysis. After analysis, the investigator will be able to request the DNAs from all recombinant animals in the interval containing the marker. These DNAs will allow the investigator the opportunity to map other closely linked markers or help in constructing a physical map of the interval. By using this approach the DNA from the reference cross will provide a long term resource (approx. 50,000 PCR amplifications per sample).

In conclusion, we have developed a resource that can be used to map genes and genetic markers to a high resolution and also provides the foundation for a future physical mapping project. This reference cross DNA panel has been anchored by 102 SSLPs. The SSLPs and the reference cross DNA panel are now available to the community. The utility of the reference cross will continue to increase as more markers are mapped and the density of the map improves. The framework map is tentative but usable, while work proceeds on completing the genetic map consisting of SSLPs and physically anchoring it to the chromosomes. The map and the reference cross will also play an important role in establishing conserved gene segments between the zebrafish genome and genomes of different organisms.

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