

A Genetic Linkage Map of a Cichlid Fish, the Tilapia (*Oreochromis niloticus*)

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ABSTRACT

We have constructed a genetic map for a tilapia, *Oreochromis niloticus*, using DNA markers. The segregation of 62 microsatellite and 112 anonymous fragment length polymorphisms (AFLPs) was studied in 41 haploid embryos derived from a single female. We have identified linkages among 162 (93.1%) of these markers. 95% of the microsatellites and 92% of the AFLPs were linked in the final map. The map spans 704 Kosambi cM in 30 linkage groups covering the 22 chromosomes of this species. Twenty-four of these linkage groups contain at least one microsatellite polymorphism. From the number of markers 15 or fewer cM apart, we estimate a total map length of ~1000–1200 cM. High levels of interference are observed, consistent with measurements in other fish species. This map is a starting point for the mapping of single loci and quantitative traits in cichlid fishes.

TILAPIA is the common name for ~70 species of perch-like fishes (family Cichlidae) native to the fresh waters of tropical Africa (Trewavas 1983; Stiasny 1991). They include the mouthbrooding genera *Sarotherodon* and *Oreochromis*, and substrate spawning *Tilapia*. These fishes have been introduced into nearly every tropical and subtropical country in the world to support the development of fresh-water aquaculture. The total estimated world production of tilapia now exceeds 659,000 metric tons per year (FAO Fisheries Statistics 1997), of which the majority consists of the species *Oreochromis niloticus*.

The genetic resources of tilapia have been poorly managed. Primary introductions of wild stock frequently consisted of a small number of individuals. These were serially distributed so that genetic problems have been passed from farm to farm. Genetic problems are of three kinds. First is the loss of pure species through mismanagement of interspecific hybridization (McAndrew 1993), a technique used to produce all-male fry which have a higher growth rate in production systems (Hickling 1960; Hulata *et al.* 1983). One popular commercial strain is thought to contain genes from as many as four species (McAndrew *et al.* 1988). A second problem is high levels of inbreeding depression. Eknath *et al.* (1993) compared four strains farmed in the Philippines with four strains newly isolated from wild populations in Africa. The best-performing strains were those most recently isolated from nature, which is consistent with the idea that domesticated strains suffer

from inbreeding depression (Tave and Smitherman 1980; Hulata *et al.* 1986; Teichert-Coddington and Smitherman 1988). Finally, there is evidence for contamination of genetically improved strains by introgression from feral species (Macaranas *et al.* 1986).

In recent years, the focus for fresh-water aquaculture has moved to a single species, *O. niloticus*, and research has begun to overcome some of the main problems associated with farming this species (Pullin and Capili 1988; Tave 1988). Large scale genetic improvement programs have been established for *O. niloticus* in Asia (Eknath *et al.* 1993), and genetic methodologies to control sex have now resulted in the reliable production of all-male fry to help overcome the problems associated with excessive fry production in on-growing ponds (Mair *et al.* 1995). Future research will aim to improve the performance and expand the environmental tolerance of this species into areas of lower temperatures and higher salinities. These developments will require a greater use of genetic markers for the management of stocks, identification of quantitative trait loci (QTL), and improvement of strains through marker-assisted selection.

Much is already known about tilapia genomes. The karyotypes of the various tilapia species are highly similar, consisting of 22 pairs with no morphologically distinct sex chromosomes. In fact, only two pairs are recognizable; the remaining 20 being similar in size and morphology (Majumdar and McAndrew 1986). At the molecular level, the genome size of several species has been measured at around 1 pg (1000 Mb), about one-third the size of many mammalian genomes. Genome size varies by up to 44% among species from 0.84 to 1.21 pg (Majumdar and McAndrew 1986), probably because of the evolution of repetitive element families. The SATA family consists of repeats ~230 bp long, and

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represents 7% of the *O. mossambicus* genome (Wright 1989). A 1900-bp sequence (SATB) makes up more than 2% of the *O. niloticus* genome (Franck and Wright 1993). A 320-bp SINE is present in 1000–30,000 copies/genome (Takahashi *et al.* 1997). Still, the overall size of the genome is small, only about twice as big as the smallest teleost genome known (Hinegardner and Rosen 1972).

Before the present study was conducted, only a few genes had been mapped in tilapia. Hussain *et al.* (1994) used meiotic gynogenetics (diploid progeny produced by suppression of the second meiotic division) to map gene-centromere distances for six allozyme loci, sex, and two color loci. Interestingly, some gene-centromere recombination fractions ranged as high as 100%, suggesting very high levels of interference. Similar phenomena have been reported for salmonids (Allendorf *et al.* 1986) and common carp (Komen 1990), and it has been suggested that the phenomenon might be general for fish. However, the occurrence of double recombinants and linkage groups greater than 100 cM in the zebrafish (Johnson *et al.* 1995) demonstrate that double recombinants are possible in teleosts. Estimation of the level of interference is critical to establishing total map lengths, because the various map functions make different interference assumptions to correct the observed fraction of recombinants for unobserved double recombination.

The goal of our study was to develop a comprehensive map of *O. niloticus* using DNA polymorphisms, which might be suitable for analysis of single loci and quantitative traits. Our approach was to study the segregation of these polymorphisms in the haploid progeny of a single female *O. niloticus*.

MATERIAL AND METHODS

Haploid gynogenesis: Milt was collected from *O. niloticus* into glass capillary tubes. A small volume was examined using a light microscope to ensure that the sperm were not yet activated. Sperm concentration ranged from 10^8 to 10^9 sperm/ml milt. For irradiation, the milt was diluted to 2.5×10^7 sperm/ml (Hussain *et al.* 1993) in modified fish Ringer solution (0.1 M NaCl; 40 mM KCl; 1.4 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$; 2 mM NaHCO_3 , pH 8.0). 1.0 ml diluted milt was placed into a small Petri dish and irradiated with a UV dose of 290–295 Wcm^{-2} for 2 min using a 254-nm lamp.

The *O. niloticus* female was stripped by hand and the 600–1000 eggs divided into batches of 150–200 in Petri dishes. The eggs were washed with filtered aquarium water and then 2.0 ml irradiated milt solution was added to each batch of eggs and water. Eggs were placed in incubators with a water temperature of 28° (Rana 1986). Dead eggs were removed daily. Embryos were collected 2–3 days posthatching, at which point 75 haploids were recovered. Survival varied from 0 to 10% among the several broods collected for the study.

Genomic DNA extraction: Embryos were overdosed with anaesthetic (4-aminobenzoic acid ethyl ester; Sigma Chemical, Dorset, UK). With the aid of a binocular microscope, the yolk sac was removed using watchmaker's forceps and the embryos placed into individual sterile 1.5-ml microcentrifuge tubes containing 150 μl TEN buffer (100 mM Tris-HCl, pH 8.0; 10

mm EDTA; 250 mM NaCl), 10 μl 20% SDS, and 5 μl proteinase K (10 mg/ml stock). Tubes were placed in a water bath at 37° overnight or at 55° for a few hours. Two phenol and one chloroform/isoamyl alcohol (24:1) extractions were carried out. DNA was precipitated using isopropanol. Pellets were washed in 70% ethanol, dried, and resuspended in 30 μl autoclaved dH_2O . Approximately 1 μg purified DNA was obtained from each embryo.

Microsatellite markers: The majority of microsatellite loci scored consisted of 139 di- and tri-nucleotide repeats isolated from an enriched *O. niloticus* genomic DNA library (Lee and Kocher 1996). An additional six loci isolated from *O. shiranus* (Ambali 1996) were also tested. Four markers isolated from Lake Malawi haplochromines were examined, including two loci from *Pseudotropheus zebra* (Parker and Kornfield 1996), and two from *Melanochromis auratus* (Kellogg *et al.* 1995).

Typing of microsatellites: Genotypes were obtained by automated sizing of fluorescently-tagged alleles amplified via PCR. We used a 25 μl reaction volume containing 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 2.4 mM MgCl_2 , 0.16 mM each dNTP, and 0.16 μM each primer, to which we added 20 ng of haploid genomic DNA. The PCR conditions were as follows: 95° for 1 min, 50–58° for 2 min, 72° for 2 min, and 25–30 thermal cycles, according to the efficiency of amplification. For multiplexing, another pair of primers labeled with a different fluorescent dye was added into the PCR reaction under the same conditions. For analysis, 1 μl from as many as three different PCR reactions were combined into a new tube and dried in a speed-vac. The pellet was resuspended with both 0.3 μl GeneScan 500 Tamra (Applied Biosystems Inc., Foster City, CA) and 2.7 μl formamide-loading buffer. After denaturation at 90° for 2 min, the entire solution was loaded on a 6% acrylamide gel on an ABI 373A automated DNA sequencer. ABI GeneScan software (ver. 2.02) was used to analyze the genotypes of the microsatellite loci.

AFLP markers: We used the Perkin-Elmer (Norwalk, CT) AFLP plant mapping kit (Rev. A) to implement the technique of Vos *et al.* (1995). A total of 250 ng haploid genomic DNA was used in the initial ligation step. For the selective amplification step, we tested 22 primer pairs on a panel of six haploid progeny. 1 μl of each selective amplification product, together with the GeneScan 500 Rox size standard, was loaded in each lane of a 6% gel on the automated sequencer. The number of variable bands for each primer combination is shown in Figure 1. The frequency of variable bands per primer pair ranged from 0 (ACT+CAC and ACC+CAG) to 15 (AGG+CTT). Those primer combinations generating more than five variable markers were selected for typing the remaining 35 haploids. These primer pairs were [*EcoRI*+*MseI* (number of variable bands)]: ACT+CTA (7), AGG+CTG (10), ACA+CAA (11), AGG+CTT (15), ACA+CAC (10), ACT+CAT (13), AGC+CTA (10), ACC+CAA (6), ACT+CAG (11), AGC+CAT (6), AGG+CTA (7), and AGC+CAG (9).

Linkage analysis: We used the Macintosh porting (ver. 2.0) of MAPMAKER (Lander *et al.* 1987) to identify linkage groups and determine marker order. Genotype data were entered in both phases to satisfy the requirements of the software. An initial grouping of markers was performed with a LOD cutoff of 3.0. The sequence of markers in each group was determined with the order command, and checked with the ripple command. Because of the high levels of interference observed, final map distances were calculated using the Kosambi function (Ott 1991).

RESULTS

Genotypes: The parent female and five haploid progeny were screened for a total of 149 microsatellites. The

		MseI primer							
		CAA	CAC	CAG	CAT	CTA	CTC	CTG	CTT
EcoRI primer	AAC	1	2	3	4	5	6	7	8
	AAG	3 9	10	11	12	13	14	15	16
	ACA	<u>11</u> 17	<u>10</u> 18	19	20	21	22	23	24
	ACC	<u>6</u> 25	3 26	0 27	28	29	30	31	32
	ACG	33	2 34	2 35	1 36	37	38	39	40
	ACT	41	0 42	<u>11</u> 43	<u>13</u> 44	<u>7</u> 45	46	47	48
	AGC	49	50	<u>9</u> 51	<u>6</u> 52	<u>10</u> 53	4 54	4 55	56
	AGG	57	58	59	60	<u>7</u> 61	4 62	<u>10</u> 63	<u>15</u> 64

Figure 1.—Number of variable bands for various selective primer combinations for AFLP analyses. The number of polymorphic bands is given for each of the 22 primer pair combinations examined. The 12 most polymorphic loci (underlined) were scored in the 41 haploid progeny. Numbers in the lower-right corner of each box define the AFLP locus names used in Figure 2.

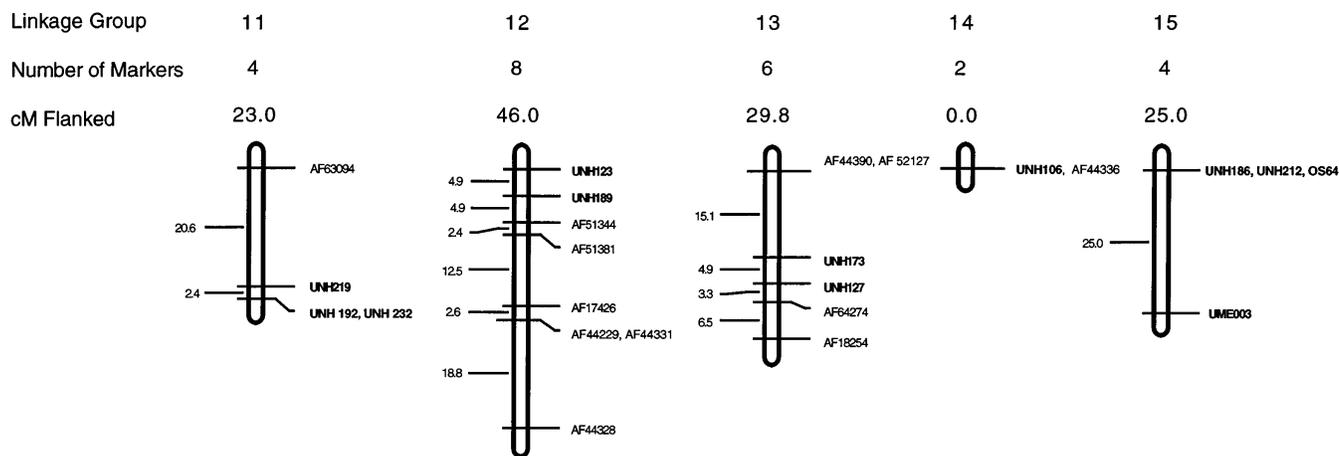
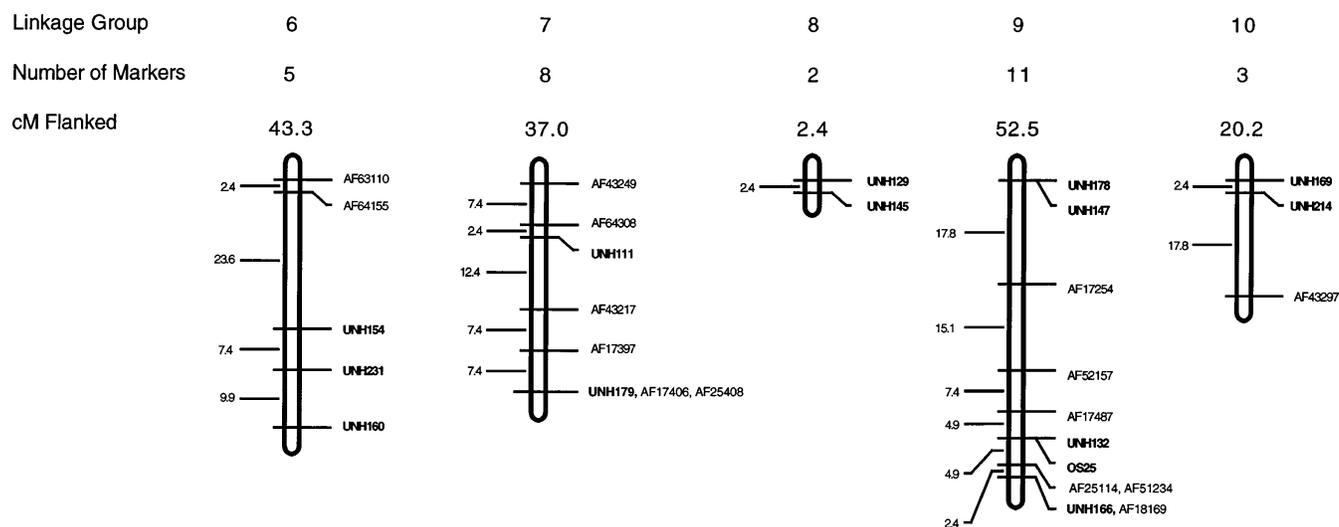
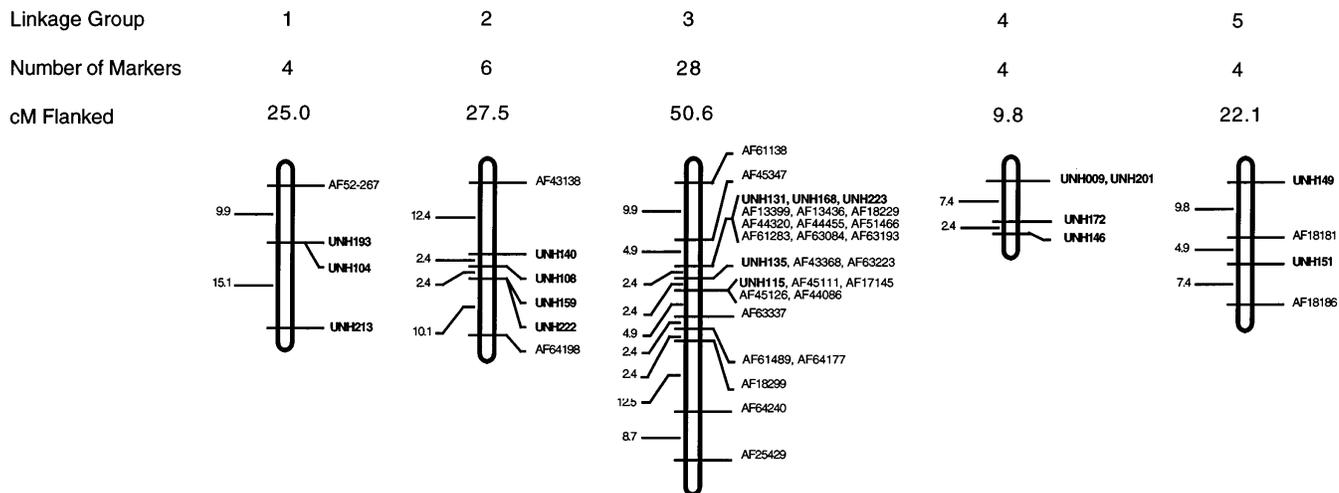
mother was heterozygous for 62 (42%) of these markers. An additional 36 haploids were scored for these 62 microsatellites. We also scored the 41 haploids for 12 AFLP primer combinations, which identified 112 AFLPs. Of these 112 presence/absence polymorphisms, we suspect that nine are alternate phenotypes of another locus. For example, five pairs of completely linked loci showed differences in length in opposite phase, suggesting that we have identified codominant alleles of a restriction site variant. Four pairs of completely linked loci showed bands of different size in the same phase, possibly because of multiple priming of the same locus. We conservatively estimate the number of polymorphic bands indicative of unique genetic loci as 103 (92% of the polymorphic bands). The final data set consisted of genotypes for 62 microsatellites and 112 AFLPs for 41 haploid progeny of a single female.

A preliminary linkage map was constructed and the genotypes for each individual examined for instances of double crossovers, which are sentinels for error in a dataset (Lincoln and Lander 1992). The electropherograms for all 32 double crossovers were reexamined, and scoring or transcription errors were detected for 16 genotypes. Correction of these errors led to a decrease in map length of ~118 cM (14%). We estimate that the original data set of 7000 individual genotypes contained less than 1% error, and that the remaining error is less than 0.5%. The final linkage map is based on a total of 268 inferred crossovers, including 19 double and 2 triple crossovers.

Linkage map: Overall, 93.1% of the markers tested showed detectable linkage to another marker. 59 of the

62 microsatellites (95%), and 103 of 112 (92%) of the AFLPs, were detectably linked to another polymorphism. The final linkage map consists of 30 linkage groups spanning 704 cM (Figure 2). A total of 162 polymorphisms are included for an average spacing of 4.3 cM. The size of the linkage groups range from 0 to 73.6 cM (mean: 23.5 cM). In Figure 2, markers are shown in the order that maximized the LOD score but it should be noted that the relatively small size of the family (41 meioses) makes the assignment of marker orders within 5 cM intervals tentative. The number of markers per linkage group varies from 2 to 28, with an average of two microsatellites and 3.4 AFLP markers per group. Twenty-four linkage groups contain at least one microsatellite polymorphism.

Segregation distortion: Because of the high mortality of haploid embryos, we were concerned about the potential for segregation distortion of markers linked to deleterious alleles. A total of 14 markers (8%) showed significant segregation distortion. The most significantly distorted markers appeared on the same linkage group (25), and four markers on that linkage group arose from a single AFLP primer pair (AF64). We suspect that this segregation distortion arises from anomalous amplification of these markers, and that the actual length of this linkage group is much shorter than it appears in Figure 2. Another suspicious cluster appears on linkage group 12, including three markers from AFLP primer pair AF44. If the six loci showing significant segregation distortion on linkage groups 12 and 25 are removed from the analysis, less than 5% of the remaining markers show significant distortion, as ex-



Continued

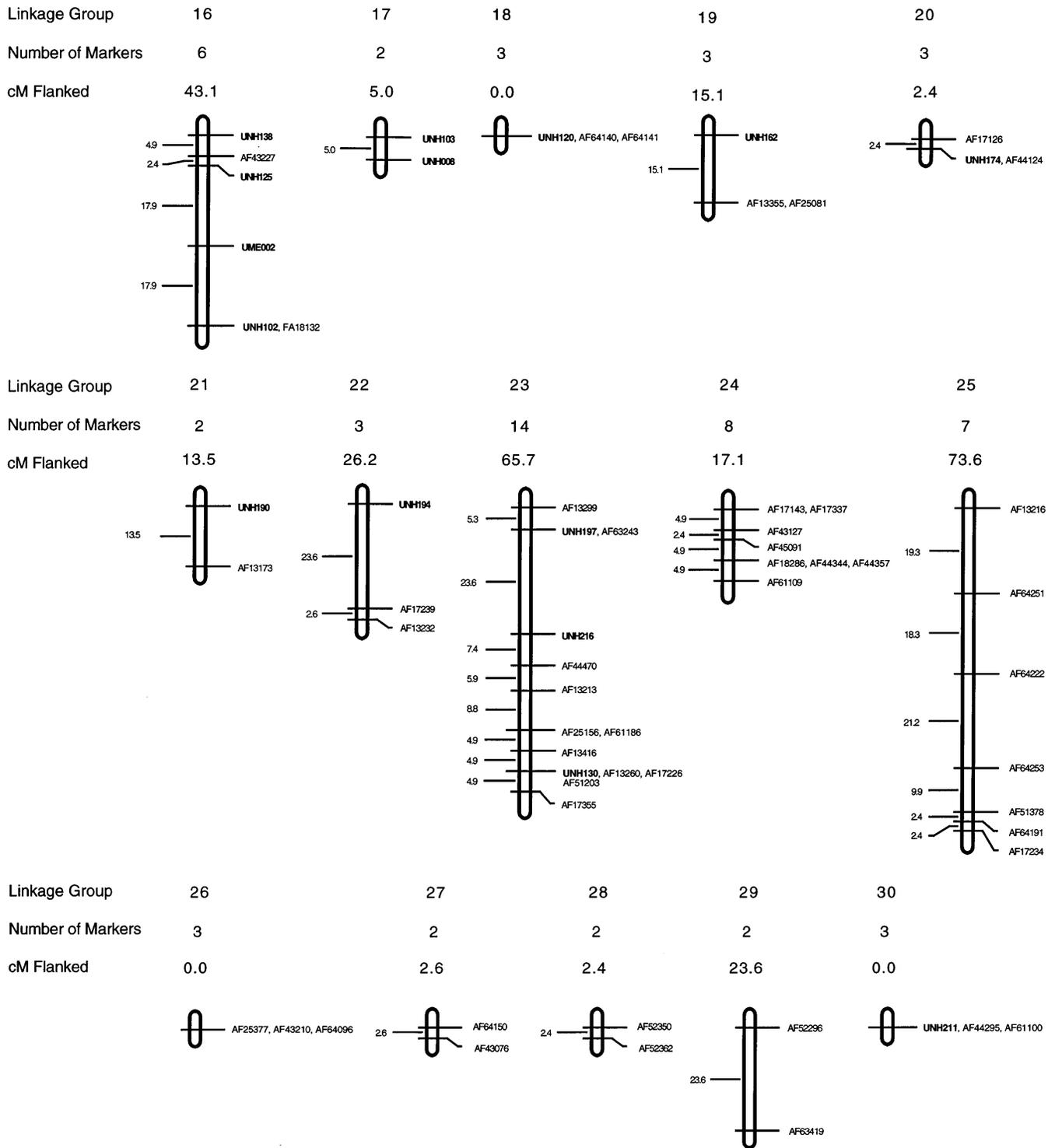


Figure 2.—A genetic map of *Oreochromis niloticus*. Microsatellites are coded by laboratory: UNH, University of New Hampshire; UME, University of Maine; OS, *Oreochromis shiranus* (A. Ambali, personal communication), followed by 2–3 digits identifying the particular marker. AFLP makers are designated AF followed by 2 digits, which identify the primer combination used (see Figure 1), followed by 3 digits which indicate the approximate size in base pairs of the scored fragment. The latest updates of this map can be viewed on the World Wide Web (<http://tilapia.unh.edu>).

pected by chance. There is no tendency for the remaining markers exhibiting segregation distortion to cluster in the linkage map.

Interference: We estimated interference by dividing the 10 largest linkage groups in half, and calculating the observed and expected frequency of double recom-

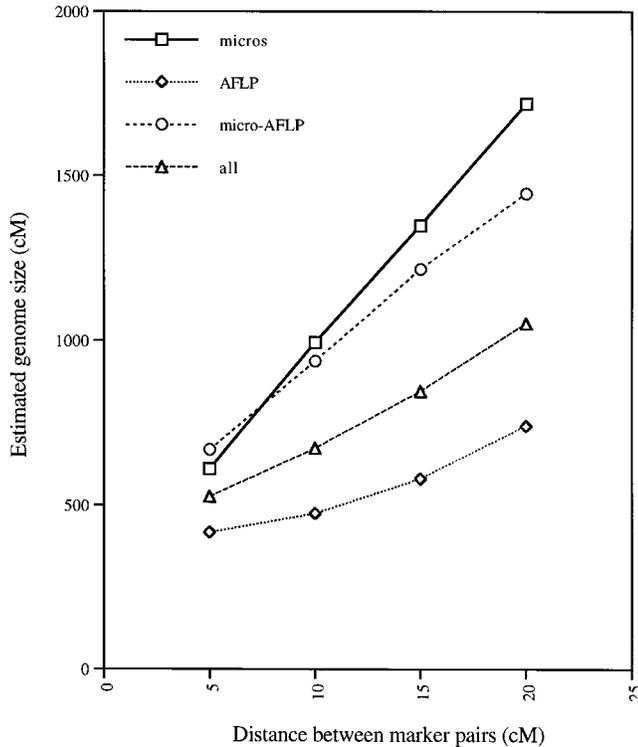


Figure 3.—Estimates of genome size derived from the proportion of markers linked at 5, 10 or 15 cM. Estimates were computed separately for microsatellites, AFLPs, microsatellite/AFLP pairs, and for all markers.

binants for each. We observed 11 double crossovers, where 24 were expected, leading to an interference estimate of 0.54 (Griffiths *et al.* 1993). This may be an underestimate of the interference, because some of the double crossovers could be on opposite chromosome arms, and because there may be some error remaining in our data.

Estimates of genome size: Hulbert *et al.* (1988) suggest that the ultimate map length can be estimated by observing the proportion of locus pairs linked at specific distances, and comparing this to an expectation based on the assumption that the loci are distributed randomly across the map. We performed these calculations separately for each marker type at four distances (Figure 3). When we analyzed the proportion of pairs exhibiting less than 5% recombination, all combinations of marker pairs gave similar estimates of genome size, ranging from 412 cM for the AFLP to 668 cM for AFLP/micro pairs. These estimates are all smaller than the spanned length of our map. For larger intervals, the estimates are less consistent, and for recombination fractions of 20%, the genome size estimates range from 740 to 1719 cM. The estimates derived from pairs of AFLP markers are consistently smaller than those for pairs of microsatellites.

DISCUSSION

Mapping with haploids: Lie *et al.* (1994) put forward the strategy of mapping through haploid gynogenesis.

The major advantage of this approach for our study was the simplicity of genotyping haploid material. The haploid material made the interpretation of the AFLPs much more straightforward.

One concern is the possibility that deleterious alleles will cause segregation distortion in the haploids. Lie *et al.* (1994) demonstrate that the presence of a lethal allele does not influence the observed recombination fraction. In our study, it appears that the most serious instances of segregation distortion arose from problems typing the AFLP markers, not from deleterious alleles.

The major disadvantage to mapping with haploids is that few phenotypic traits can be measured. Marker information is limited to PCR-amplifiable DNA polymorphisms. Thus, we have no information on the map locations for genes encoding allozymes, color or sex of the animal. In this respect, it might be useful to double the haploids through suppression of the first mitotic division (Mair 1993) to grow the animals to a larger size.

Interference: The short length of fish chromosomes may inhibit recombination and generally allow only one obligate crossover per chromosome arm. Previous work has suggested that high levels of interference are typical of fish genomes (Thorgaard *et al.* 1983). Consistent with this idea, gene-centromere recombination rates of 100% have been observed in tilapia meiogynes (Husain *et al.* 1994). High levels of interference are also observed in the zebrafish genome (Kauffman *et al.* 1995), but double, and even triple, crossovers are observed, and some chromosome arms have lengths greater than 100 cM (Johnson *et al.* 1995, 1996).

Cytogenetic studies of tilapia suggest a single chiasma per chromosome, except for the largest chromosome, which appears to have chiasmata in both arms (Majumdar and McAndrew 1986). Our data indicate high levels of interference over relatively large map distances. Larger family sizes will be needed to describe the shape of the interference function with distance (*e.g.*, Amati and Meselson 1965).

Length of the genome: One of the goals of this study was to estimate the map length of the tilapia genome. Although the size of the genome is $\sim 1 \times 10^9$ bp, it is well known that genetic distance corresponds poorly to physical distance. Our map currently spans 704 cM, but we suspect at least two linkage groups (12 and 25) are artificially expanded by AFLP genotyping errors. If we remove these markers, the total spanned length might be reduced to as little as 625 cM. At the same time, the probability that a new marker will be detectably linked to the map is already 93%, even with our relatively small family size. This result suggests that the map will not expand dramatically as more markers are added.

Our estimates of the total map length from the proportion of locus pairs linked at various distances were not consistent, suggesting that the assumptions of the approach are violated. The estimates from the AFLP markers are smaller than those for the microsatellite markers, probably because codominant alleles were

scored as separate loci, inflating the proportion of linked pairs. The positive slope of the lines in Figure 3 could be an indication that the markers are clustered in the genome. More probably, it is a consequence of the high levels of interference observed in this genome. Interference suppresses recombination, thus increasing the proportion of pairs that appear to be closely linked. This reduces the estimate of genome size derived from closely spaced markers. The same phenomenon reduces the proportion of pairs linked at longer distances, thus increasing the estimates of genome size. Although final estimation of genome size will have to wait until the map is saturated with markers and the interference phenomenon is more completely characterized, the current data suggest the total map length will be ~ 1000 cM.

Strategies for QTL mapping: Microsatellites have become the preferred marker for animal gene mapping because of their high heterozygosity and ease of typing via PCR. AFLP is a new approach that offers rapid marker development and typing. Because we used both techniques in developing our map, we offer the following comments on the methods:

Typing of microsatellite markers is slow relative to techniques such as AFLP. In our hands, only 4–5 microsatellite loci can be effectively multiplexed on the same gel. In contrast, we routinely score 12 polymorphisms from some AFLP primer combinations, and we expect to be able to load at least two such combinations per lane. For the rapid construction of a dense genetic map suitable for QTL analysis in any particular cross, AFLP may be the fastest route.

We caution that speed comes at a price. We encountered far more genotyping errors with AFLPs. While in our study these appear limited to a few primer combinations, we expect that accurate scoring will become more difficult when attempting to score diploid genotypes. In addition, AFLP markers are typically dominant, which will reduce the informativeness of AFLP markers in most crosses. AFLP markers will likely be much less transportable among labs, crosses, and species. Alignment of AFLP maps produced from different crosses may be extremely difficult.

It may be possible to use a mixed strategy for mapping QTL. High-density AFLP maps may be anchored with a much smaller set of microsatellite loci. We have already mapped at least one microsatellite on 24 of the 30 linkage groups, and it seems likely that we have mapped at least one microsatellite on each chromosome. These anchor loci will allow comparison of AFLP maps produced for QTL analyses in different laboratories.

The next step: We have several goals in continuing this line of research. The first is the identification of QTL in different strains of tilapia, which might be usefully combined to produce a better fish for aquaculture. The map we have constructed is adequate for that purpose. Although we cannot expect that all 62 of these microsatellite markers will be variable in other crosses, we will continue to score the other 84 microsatellites

already characterized, and hope to eventually incorporate all of them into the map. Inclusion of 50–60 microsatellites in each experimental cross will be sufficient to identify homologous chromosomes. Marker density is most conveniently increased in each cross through the typing of AFLP markers.

A second goal is to use these genetic markers to characterize germplasm resources of tilapia. Preliminary work suggests that microsatellites are a useful way to estimate heterozygosity of stocks, and will be very useful for tracking parentage in selection experiments. Preliminary AFLP data suggests that this technique will be useful for classifying tilapia strains to species, or identifying their probable hybrid origins.

Finally, we plan to extend our mapping efforts to other groups of cichlids, particularly the species flock of Lake Malawi haplochromines (Kocher *et al.* 1993). A large proportion of our tilapia markers also amplify Lake Malawi cichlids, and it will be interesting to determine the extent of synteny with this group. A genetic map for these fish would allow QTL mapping of traits associated with speciation and adaptive radiation of these fishes.

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