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Breeding new strains of tilapia: development of an artificial center of origin and linkage map based on AFLP and microsatellite loci

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Abstract

Based on ideas from plant breeding and the opportunities offered by molecular biology, a program was initiated in 1995 to derive genetically superior tilapia from a synthetic stock (artificial center of origin, ACO) produced by inter-crossing five groups of fish: *Oreochromis niloticus* [wild-type (*On*) and red (R*On*) strains], *O. aureus* (*Oa*), *O. mossambicus* (*Om*), and *Sarotherodon galilaeus* (*Sg*). Three-way cross families (3WC) and four-way cross families (4WC) have been produced, so that all four species are represented in the ACO. A genomic map has been created for each of the parents in an *Om* × (*Oa* × R*On*) family using microsatellite and AFLP

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(amplified fragment length polymorphism) DNA markers. The female (*Om*) parent had a total of 78 segregating markers (17 microsatellites, 61 AFLPs). Of these, 62 (13 microsatellites, 49 AFLPs) were linked in 14 linkage groups covering a total of 514 centimorgans (cM). The first generation (F_1) hybrid male parent had a total of 229 segregating markers (62 microsatellites, 167 AFLPs), of which 214 (60 microsatellites, 154 AFLPs) were linked in 24 linkage groups covering a total of 1632 cM. The construction of these maps is a key step in a molecular marker-assisted breeding program to detect quantitative trait loci (QTL) for cold and salinity tolerance and carcass quality in tilapia. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Tilapia; *Oreochromis*; Genetic map; AFLP; Microsatellite; Cichlidae; Interspecific hybridization

1. Introduction

The use of interspecific composite (or complex) crosses, an established practice in plant breeding, is a means of achieving wide genetic and phenotypic variability. The method is based on the creation of an artificial center of origin (ACO) via composite interspecific crosses. The ACO contains wide genetic diversity and presents opportunities for genes to recombine and interact with other genes originating from different species; combinations and interactions that are impossible in any of the pure species. An adaptation of this method, termed multiple re-speciation (MRS), recently was applied to develop new cultivars of carnations (Umiel, 1993).

We have constructed a tilapia ACO, exploiting the relative ease of making interspecific hybrids with these species (Wohlforth and Hulata, 1983). The ACO involved four tilapiine species: *Oreochromis niloticus* (wild-type (*On*) and red (R*On*) strains), *O. aureus* (*Oa*), *O. mossambicus* (*Om*), and *Sarotherodon galilaeus* (*Sg*).

Genetic maps have been constructed for a number of fish species including zebrafish, *Danio rerio* (Postlethwait et al., 1994; Johnson et al., 1996); rainbow trout, *Oncorhynchus mykiss* (Young et al., 1998); and Nile tilapia, *O. niloticus* (Kocher et al., 1998). In order to best utilize the unique genetic material produced in the creation of the ACO, we are producing linkage maps from several three-way and four-way crosses (3WC and 4WC, respectively) produced as part of the breeding program. These maps provide linkage information for markers that are segregating directly in the multi-specific crosses, they further develop the existing map of the *On* genome (Kocher et al., 1998), and they establish a framework for the detection of quantitative trait loci (QTL) for traits of importance to the culture of tilapia. These maps will also be used to evaluate the genetic composition of the strains derived from the ACO. Here we report on the results of the breeding program that created the ACO and linkage maps from a 3WC family containing genetic material of *Oa*, *Om*, and R*On*.

2. Materials and methods

2.1. Breeding program

Four different species of tilapia were used for producing the composite population (Table 1). Breeding of tilapias was carried out at the Department of Aquaculture,

Table 1
Tilapia species and strains used in the breeding program of this study

Species	Abbreviation	Origin
O. aureus (Steindachner)	*Oa*	Local Israeli Mehadrin strain, described by Hulata et al. (1993)
O. niloticus (Linnaeus)	*On*	Ghana strain (Mires, 1977; Hulata, 1988)
Red *O. niloticus*	R*On*	Originated in Lake Manzala, Egypt (McAndrew et al., 1988), and obtained from the Institute of Aquaculture, University of Stirling, Scotland
O. mossambicus (Peters)	*Om*	Natal, South Africa (Hulata, 1988)
S. galilaeus (Linnaeus)	*Sg*	Endemic species originating in Lake Tiberias (Kinnereth, Sea of Galilee) (Ben-Tuvia, 1959; Goren, 1974)

Agricultural Research Organization, Bet Dagan, Israel, except where otherwise stated. Parental fish were individually tagged using colored and numbered plastic discs, attached to the fish on nylon threads pushed through the dorsal muscle and knotted. Parents of all crosses retained for further study were PIT tagged (Destron-Fearing, St. Paul, MN, USA) and placed in holding tanks. Starting with the parental species, two-way crosses (2WC) were made to produce first (F_1) and second (F_2) generation populations. Some of the 2WCs were crossed to a third species to produce a series of 3WC, or intercrossed to create 4WC.

Reproduction procedures were similar to those described in Rosenstein and Hulata (1994), except that eggs removed from the buccal cavities of females were transferred to hatching jars to complete incubation. The *S. galilaeus* × *Oreochromis* sp. F_1 hybrids were produced by artificial fertilization (Don and Avtalion, 1986; Yeheskel and Avtalion, 1988) at the Laboratory of Fish Immunology and Genetics, Bar-Ilan University, Ramat Gan, Israel. To produce the artificially fertilized groups, a batch of eggs of a single *S. galilaeus* female was divided into four sub-samples, each fertilized with sperm collected from males of the four *Oreochromis* stocks. Samples of the F_1 progeny were transferred to Bet Dagan for use in the breeding program. 3WCs and 4WCs were obtained from these F_1 fish by natural spawning.

2.2. Linkage mapping

2.2.1. Tissue

Sixty-three 3WC progeny of a single mating between an *Om* female and an F_1 (*Oa* × R*On*) male were chosen for evaluating joint segregation of microsatellite and amplified fragment length polymorphism (AFLP) markers. Fin tissue (1–4 cm^2) was collected from adult fish of each parental species, including all parents of the progeny group contributing to the ACO, and stored in 95% ethanol. The tissue of progeny was obtained from newly hatched fry (10–14 days old) and placed in 95% ethanol for storage. DNA from a 0.7-cm^2 piece of fin or an entire fry was extracted using the CTAB phenol/chloroform protocol of Saghia-Maroof et al. (1984) and Doyle and Doyle (1987), as modified by Grewe et al. (1993).

2.2.2. Microsatellites

Fluorescently labeled microsatellite primer pairs ($n = 133$) developed from an *On* genomic library (Lee and Kocher, 1996) were purchased from Research Genetics (Huntsville, AL, USA). These were screened for the ability to amplify alleles and demonstrate polymorphism in the mapping family and in individuals from each of the five ''pure'' species used in the breeding program. PCRs were performed in 10 μl volumes in a PTC-100-96V Thermocycler (MJR; Watertown, MA, USA). The reaction conditions were as follows: Gibco 1× PCR buffer (20 mM Tris–HCl pH 8.0, 50 mM KCl), 2.4 mM $MgCl_2$, 160 μM each dNTP, 0.2 U *Taq* polymerase, 0.16 μM each primer, and 10 ng of template DNA. An initial screen of the primers for amplification and polymorphism was done at 28 cycles of 94°C for 1 min, 50°C for 2 min, and 72°C for 1 min. The annealing temperature was adjusted for each locus as necessary to produce scorable amplification product.

2.2.3. AFLP protocol

The use of AFLP markers was based on a modification of Vos et al. (1995). As described below, visualization of bands in gels and data collection utilized a fluorescent scanning system.

2.2.3.1. Digestion–ligation. Genomic DNA (200 ng) was restricted for 1 h at 37°C in a 200-μl tube containing 2 U each of *Eco*RI and *Mse*I (New England Biolabs; Beverly, MA, USA) and 1× NEBuffer 2 (10 mM Tris–HCl, 10 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT, 100 μg/ml BSA, pH 7.9) in a total volume of 16 μl. After digestion, 4 μl of a ligation mixture (20 pmol of *Mse*I adapter, 2 pmol of *Eco*RI adapter, 1 mM ATP, 4 U T4 DNA ligase (New England Biolabs), 100 μg/ml BSA, and 1× NEBuffer 2) was added. The reaction mixture was incubated at 25°C for 12–16 h, and then diluted 10-fold with 180 μl of TLE (10 mM Tris–HCl pH 8.0, 0.1 mM EDTA).

2.2.3.2. Pre-amplification. A pre-amplification step was used to reduce the number of possible bands to amplify, and to create a virtually unlimited supply of digestion–ligation template. Primers complementary to each adapter sequence with a one-base extension were used to amplify 1/16 of the total restriction fragments created in the digestion–ligation above. The template, 5 μl of diluted digestion–ligation product, was amplified using 30 ng each of *Eco* + G and *Mse* + C primers in a 20-μl reaction containing Gibco 1× PCR buffer (20 mM Tris–HCl pH 8.0, 50 mM KCl), 1.5 mM $MgCl_2$, 200 μM of each dNTP, and 0.4 U of *Taq* polymerase. The template was amplified for 24 cycles of: 94° for 30 s, 56°C for 1 min, and 72°C for 1 min. The PCR product was diluted 10-fold with 180 μl of TLE, and stored at 4°C.

2.2.3.3. Selective amplification. It was determined empirically that a two-base extension on the fluorescein-labeled *Eco*RI primer and a three-base extension on the *Mse*I primer produced the optimum number of bands to be easily and unambiguously analyzed on a gel. Selective amplifications were carried out in a 20-μl reaction where 5 μl of diluted pre-amplification product were amplified using the same reaction conditions as for the pre-amplification, except that the amount of primers used was 5 and 30 ng for the *Eco*

and *Mse* primer, respectively. Reactions were amplified with 30 s denaturing at 94°C, followed by 30 s annealing at 65°C and 1 min extension at 72°C. This cycle was repeated 11 more times reducing the annealing temperature 0.7°C each cycle. The cycle was repeated a final 24 times with the annealing temperature held at a constant 56°C.

2.2.4. Electrophoresis

After amplification, an equal volume of loading buffer (98% formamide, 10 mM EDTA) was added to the amplification products of both the microsatellite reactions and the AFLP reactions. The reactions were denatured at 95°C for 2 min, then snap-cooled on ice for at least 5 min prior to loading on a 5% polyacrylamide (19:1 acrylamide:bi-sacrylamide), 7.5 M urea denaturing gel (20 cm × 32 cm × 0.4 mm). Microsatellite products (48 samples; 2.5 μl each) were loaded on the gel and run at 35 W for 25 min. Then another 48 reactions from the same locus were loaded and the gel was run for 30 more minutes. AFLP products (48 samples) were loaded and the gels were run at 35W (50°C) between 1 and 1.5 h, depending on the fragment sizes for a particular primer combination.

2.2.5. Scoring and analysis

Each band on the AFLP gel was scored as band presence $(+/+, +/-)$ as one allele, or absence $(-/-)$ as the alternate allele. Although it has been reported possible in other studies (van Eck et al., 1995; Vos et al., 1995), we did not feel confident enough distinguishing differences in band intensities between homozygotes $(+/+)$ and heterozygotes $(+/-)$ to score the AFLP bands as codominant markers. For all loci scored, the approach used was to score only loci for which one parent was scored as band-present and the other parent was scored as band-absent. All loci scored were checked for segregation distortion using a χ^2 for goodness of fit to a 1:1 genotype ratio ($p < 0.05$). Twenty-five loci that showed a significant deviation were not included in further analyses.

Bands were sized using ''FragmenNT Analysis'' (Molecular Dynamics; Sunnyvale, CA, USA) and genotypes were scored by the presence or absence of bands by eye. Linkage was analyzed using Mapmaker 3.0 (Lander et al., 1987). The LOD criterion for determining linkage was taken as 3.0 and the map units used were Kosambi centimorgans.

The linkage phase for some of the loci from parental fish used to make the cross was not known. Therefore, data were entered into Mapmaker first as the bands appeared on the gel (band present = H, band absent = A), and as the reciprocal data set by changing A's to H's and H's to A's. This process created two identical but reciprocal sets of linkage groups, so only one set was used for further study.

Data also were organized using ''Map Manager XP'' (Cudmore and Manly, http://mcbio.med.buffalo.edu/mapmgr.html), which provides graphic display of the genotypes of linked loci, and therefore enables visual inspection of recombination events. When double crossovers were observed in a small area, an indicator of possible genotyping errors (Lincoln and Lander, 1992), gels were reexamined and corrections were made in the data set if necessary. Linkage groups were drawn using the ''Draw-Map'' package (Van Ooijen, 1994).

3. Results

3.1. Breeding program

The following 2WCs (female listed first) were produced: *On* × *Oa*, *Oa* × *ROn* (1995), *Om* × *Oa*, *Sg* × *Oa*, *Sg* × *Om*, *Sg* × R*On*, *Sg* × *On* (1996), *Om* × R*On*, *On* × *Om* (1997) and *Oa* × *On* (1998). We failed to produce any hybrids with R*On* as a female, apparently due to behavioral incompatibility (Hulata et al., 1995).

In 1997, F_2 groups produced from *Oa* × R*On* hybrid progeny and the following 3WCs were produced: [*Om* × (*Oa* × R*On*)], [*Om* × (*On* × *Oa*)], [*On* × (*Om* × *Oa*)], and [*Om* × (*Sg* × *On*)]. Thus, all 3WCs required for mapping the five strains of the four participating species were produced and stored in 95% ethanol for further analysis (Table 2). Some of the 3WC were difficult to produce: three spawns of [*Om* × (*Sg* × *Oa*)] produced progeny, but none were viable; seven spawns of [*On* × (*Om* × *Oa*)] were attempted, but only a few eggs hatched in only one spawn; 28 spawns of [*Om* × (*On* × *Oa*)] were obtained from two parental breeding groups, but none was viable; another breeding group produced six spawns with three producing viable offspring, two had high mortality, and one was not viable.

Most possible 4WC combinations were attempted with the following results: [(*Om* × *Oa*) × (*Sg* × *On*)], its reciprocal, and [(*Sg* × *Om*) × (*On* × *Oa*)] produced viable offspring. The 4WC [(*Sg* × R*On*) × (*Om* × *Oa*)] was difficult to produce: we had one successful spawn with only 20 surviving fish. Several spawns of [(*Oa* × R*On*) × (*Sg* × *Om*)] were not viable, and other attempted combinations for producing 4WC did not spawn at all.

3.2. Linkage maps

A linkage map was created for each of the parents in the mapping family. There were totals of 63 microsatellites and 229 AFLPs segregating in one or the other parent. The

Table 2
Summary of all 3WCs obtained, fixed in ethanol as fry and shipped to UCDavis for mapping purposes (each pool represents a different F_1 male and one to three females). See Table 1 for abbreviations

Genetic combination	Total number of fry	Number of spawns pooled
Om × (*Oa* × R*On*)	522	1
Om × (*Oa* × R*On*)	414	3
Om × (*Oa* × R*On*)	312	1
Om × (*Oa* × R*On*)	546	2
Om × (*On* × *Oa*)	523	3
Om × (*On* × *Oa*)	406	3
On × (*Om* × *Oa*)	374	2
On × (*Om* × *Oa*)	435	2
Om × (*Sg* × *On*)	693	3
Om × (*Sg* × *On*)	270	2

Table 3
The 30 primer combinations used to generate the AFLP markers. Numbers of polymorphic markers are shown for each combination

Selective extension		Female poly.	Male poly.	Total
Eco	*Mse*			
GA	GAG	3	8	11
GA	CGA	1	4	5
GA	CGC	5	9	14
GA	CGG	2	3	5
GA	CTA	0	6	6
GA	CTC	3	4	7
GA	CTG	1	11	12
GA	CTT	1	6	7
GC	CAA	4	8	12
GC	CAT	1	3	4
GC	CCC	1	1	2
GC	CCG	1	3	4
GC	CCT	3	5	8
GC	CTG	2	7	9
GG	CAT	1	5	6
GG	CCC	3	5	8
GG	CCT	0	8	8
GG	CGC	2	2	4
GG	CGG	2	1	3
GG	CGT	2	4	6
GG	CTA	4	6	10
GG	CTC	2	8	10
GG	CTT	3	5	8
GT	CCT	1	7	8
GT	CGC	0	3	3
GT	CGG	3	3	6
GT	CTA	2	8	10
GT	CTC	0	8	8
GT	CTG	3	8	11
GT	CTT	4	8	12

AFLP primer combinations used and the numbers of polymorphic loci amplified for each combination are listed in Table 3. The female (*Om*) parent had a total of 78 markers segregating (17 microsatellites, 61 AFLPs). Of these, 62 (13 microsatellites, 49 AFLPs) were resolved into 14 linkage groups covering a total of 514 cM (Fig. 1A). The male had many more segregating markers because it was an F_1 hybrid ($Oa \times \mathrm{R}On$). The male parent had a total of 229 markers segregating (62 microsatellites, 167 AFLPs). Of these, 214 (60 microsatellites, 154 AFLPs) were resolved into 24 linkage groups covering a total of 1632 cM (Fig. 1B).

3.3. Alignment of tilapia maps

Meioses in each parent are independent events, so the use of a 3WC mapping family resulted in two separate data sets, each providing a separate linkage map. A linkage map

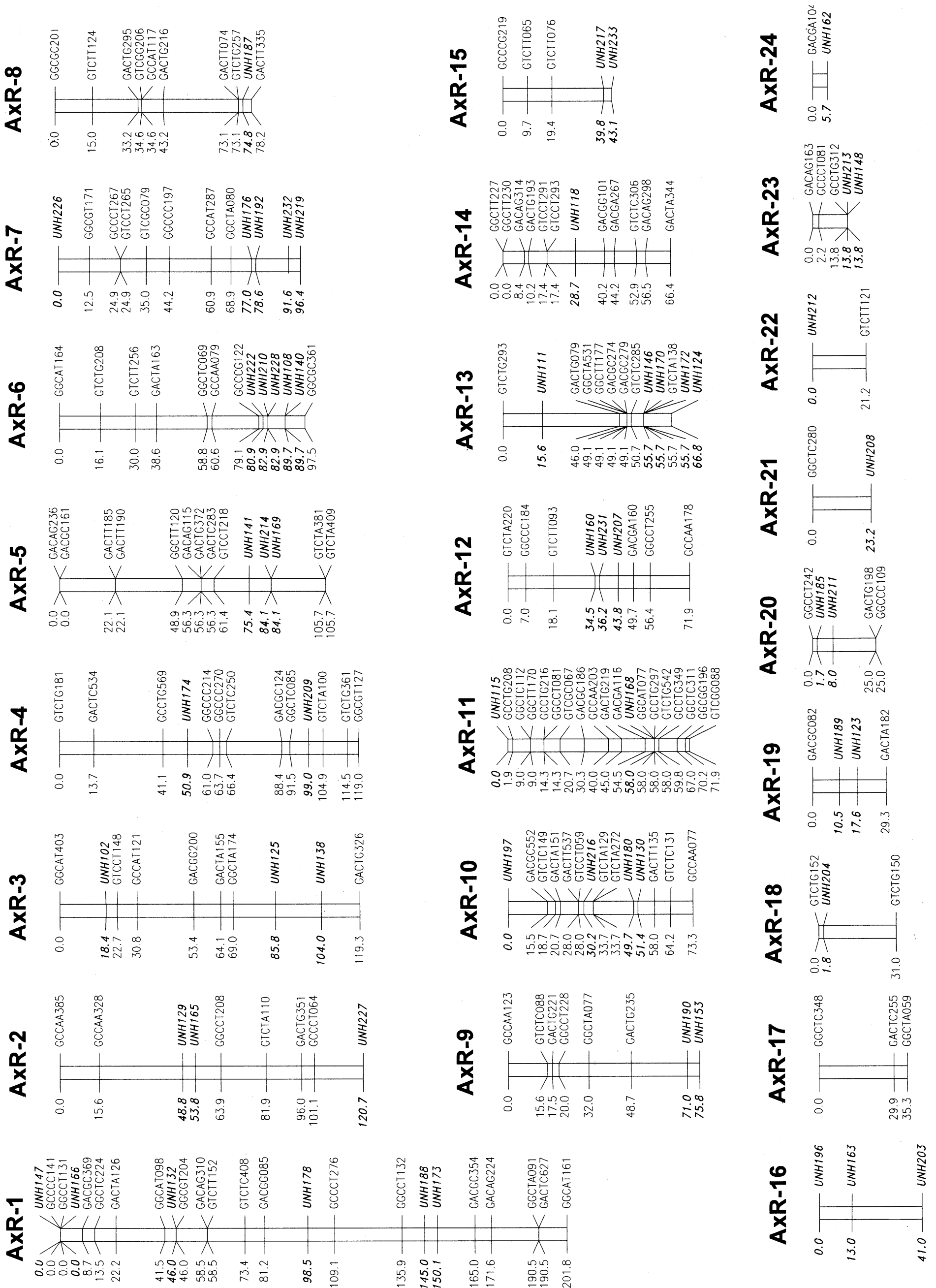

Fig. 1. (A and B) A linkage map of the male tilapia (R*On* × *Oa*) and the female (*Om*), respectively. Microsatellites are identified by ''UNH'' and a three-digit identification number, and are emphasized in bold text. AFLPs are identified by G*C**NNN, where ''G*'' refers to the *Eco*-selective nucleotides, and the ''C**'' refers to the *Mse*-selective nucleotides. Linkage groups are numbered by descending size, following the convention of a karyotype, but these linkage groups cannot be assigned yet to individual chromosomes.

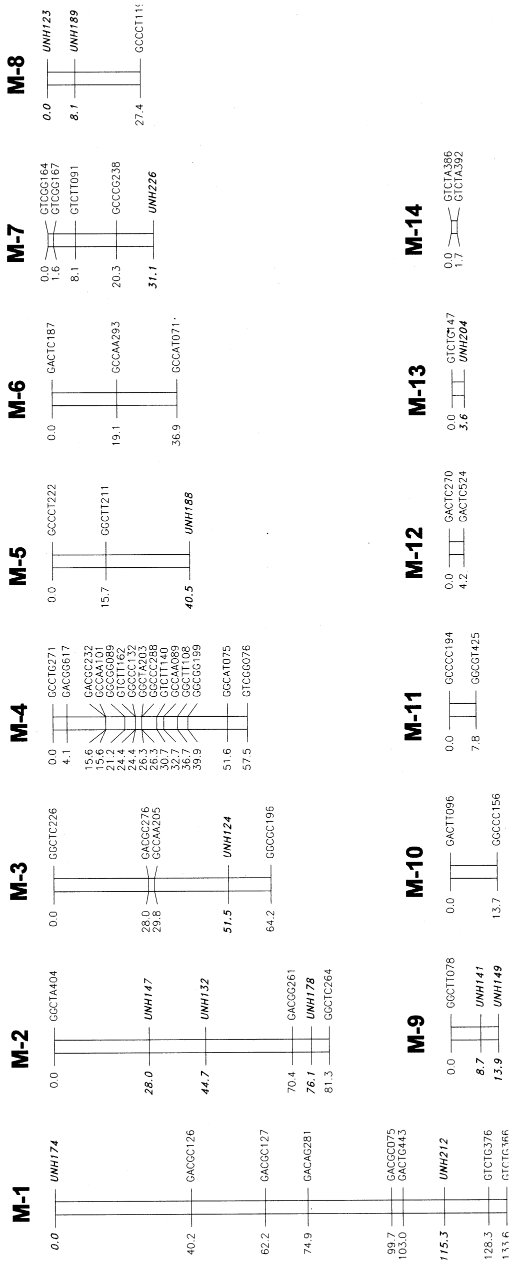

Fig. 1 (*continued*).

has been published by Kocher et al. (1998) based on haploid gynogens from a single *On* female (referred to here as the UNH map). All three of these maps have microsatellite

Table 4
Linkage group homology in three linkage maps of tilapia. ''UNH'' linkage groups from Kocher et al. (1998). ''A×R'' and ''M'' linkage groups are from the male *Oa*×R*On* and female *Om* in this study, respectively

UNH	A×R	M
1	23	
2	6	
3	11	
4	13	
5		9
6	12	
7	13	
8	2	
9	1	2
10	5	
11	7	
12	19	8
13	1	2, 5
15	22	1
16	3	
19	24	
20	4	1
21	9	
23	10	
30	20	

markers in common, which can be used as ''allelic bridges'' (Gebhardt et al., 1991) to join and align them. Linkage groups found to have syntenic regions based on at least one

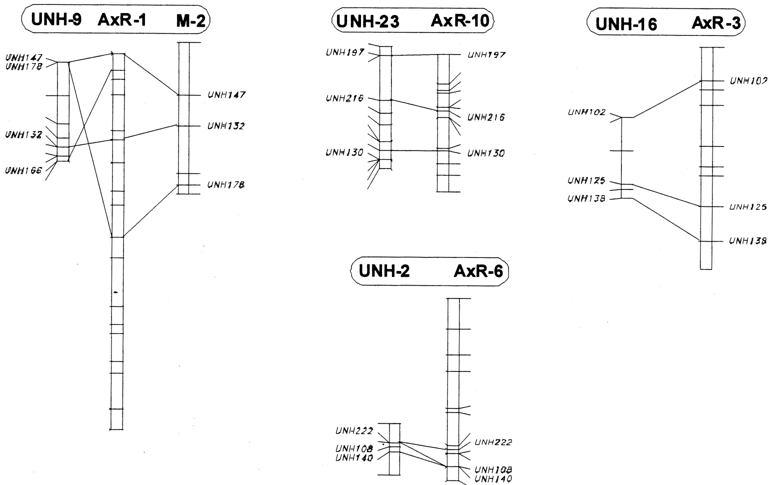

Fig. 2. Comparisons of linkage groups for tilapia showing synteny for three or more microsatellites. Linkage groups from different maps are identified as follows: ''A×R'' — male *Oa*×R*On* (this study), ''M'' — female *Om* (this study), and ''UNH'' — female *On* (Kocher et al. 1998). Note the inversion in the region between the microsatellite loci UNH-178 and UNH-166.

shared microsatellite locus are listed in Table 4. The group A × R-13 combined the groups UNH-4 and UNH-7. The group A × R-1 combined UNH-9 and UNH-13. Group M-1 combined A × R-4, A × R-22, UNH-15, and UNH-20. Alignment of linkage groups with three or more microsatellites in common resulted in four aligned sets (Fig. 2).

3.4. Species comparisons

There is evidence of at least one chromosomal inversion event among the UNH linkage group 9 and both of the maps obtained by this study (A × R-1, M-2). The region between the loci UNH-166 and UNH-178 is inverted in linkage group UNH-9 with respect to the A × R-1 and M-2 in this study.

The 63 microsatellite loci that were determined to be polymorphic in the mapping family were also tested for amplification in the five parental species used in the breeding program, by testing five individuals of each parental species. In every case, a PCR product was visible on the gel. PCR conditions needed to be optimized for several loci, but interspecific and even inter-generic amplification was demonstrated for these 63 loci.

4. Discussion

4.1. Alignment of tilapia maps

Although we were not yet able to combine all three maps into one, our efforts did further enhance the work presented by Kocher et al. (1998) by combining some previously reported linkage groups. We were unable to combine all three maps for several reasons. Many linkage groups only had one shared microsatellite, so it was impossible to assess the orientation of the markers within the group. The UNH map and the *Om* map from this study were from a female parent, while the *Oa* × R*On* map was from a male. It is possible that males and females have different recombination rates that would lead to different linkage distances between markers. In general, distances between similar markers tend to be smaller in the female map than the male map, but from one family it is premature to make any generalizations about relative recombination rates in males and females. The three maps include four different species and, while they have all been shown to have the same chromosome number, the number of arms may be different (Majumdar and McAndrew, 1986). This is due to chromosomal rearrangements, such as Robertsonian translocations or fusions, which could cause different loci to be linked in the different maps. Majumdar and McAndrew (1986) did not see any evidence of these rearrangements in the chromosomal banding patterns of seven tilapiine species, but they did not study chromosomal pairing in hybrids.

4.2. Species comparisons

Majumdar and McAndrew (1986) suggested that there might be pericentric inversions between tilapiine species that account for differences in centromere placement. The inversion shown in Fig. 2 could be evidence of such an inversion. As further evidence of an inversion, in a hybrid between *On* and *Oa* (or *Om*) the region between the loci

UNH-147 and UNH-178 would show absolute linkage because there would be no recombination in this area.

It has been proven difficult in the past to distinguish some species of tilapia based on morphology (Trewavas, 1983; Macaranas et al., 1986). This breeding study exacerbates the identification problem because interspecific hybrids are particularly ambiguous to identify based on morphology. Some of the markers used to create the map are diagnostic for distinguishing the strains used in this cross. These markers can be used to track the parentage of crosses and will be useful in tracking the species origin of alleles determined to be linked to QTL.

It should be noted that when scoring AFLP markers, we are scoring alleles and not necessarily loci. There are many cases where two adjacent polymorphisms from the same primer combination show absolute linkage on the map. It is likely that these are actually alleles at the same locus, but without sequence characterization of the bands it is impossible to reach definitive conclusions. It is less likely that two alleles at a locus would have mutations that would cause them to be amplified by different primer combinations. Roupe van der Voort et al. (1997) found that the absolutely linked loci are often alternate alleles at a single locus. The map presented here is not unusual in showing some clustering of AFLP markers, and the scoring of alleles as independent loci is a likely explanation of this observation. Roupe van der Voort et al. (1997) also showed that same-sized AFLP bands amplified from the same primer combination in different crosses were usually (95%) alleles at the same locus. This information will allow maps of further crosses of tilapias to be aligned based on shared AFLP markers as well as microsatellite markers.

This study will continue by constructing genetic maps of crosses of other species used in the creation of the ACO. These crosses will contain new polymorphic loci that will continue to add to an increasingly dense consensus map of tilapia. Our maps will be aligned with each other through the use of the same species being involved in production of different F_1 hybrid parents. Further work should also include the addition of highly conserved type I markers to the map so that the genome of tilapia can be compared to other organisms based on homologous markers. The high polymorphism of the hybrids produced in this study should overcome the concerns of low polymorphism at these types of loci. Genetic mapping of centromeres will be completed soon (Tom Kocher, University of New Hampshire, personal communication), and linkage groups will then be associated with specific chromosomes.

The maps produced for the ACO families currently are being used for determining the location of QTL for cold tolerance and salinity tolerance. Markers linked to QTL detected could be used in a marker-assisted selection (MAS) program to increase the growing range and productivity of tilapia aquaculture. Phenotypic challenges are currently underway, and the first practical application of the mapping of the tilapia genome will soon be tested.

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