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Creating and Characterizing Genetic Variation in Tilapia Through the Creation of an Artificial Center of Origin

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Creating and Characterizing Genetic Variation in Tilapia Through the Creation of an Artificial Center of Origin

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Abstract

Five stocks of tilapia [*Oreochromis niloticus* (*On*), red *O. niloticus* (*ROn*), *O. aureus* (*Oa*), *O. mossambicus* (*Om*), and *Sarotherodon galilaeus* (*Sg*)] were used to produce two-way (F_1), three-way (3WC) and four-way crosses (4WC). Three 4WC groups, containing equal representation of all four species, formed the base population for a new synthetic stock, called an “artificial center of origin” (ACO). Four genomic maps were created using microsatellite and AFLP markers, two from a 3WC family [*Om* female and (*Oa* x *ROn*) male] and two from a 4WC family [(*Om* x *Oa*) females and (*Sg* x *On*) male]. Sixty-two loci segregating from the female parent of the 3WC mapped to 14 linkage groups while 214 loci from the male parent mapped to 24 linkage groups. Similarly, 131 loci segregating from the female parent of the 4WC mapped to 26 linkage groups and 118 loci from the male parent mapped to 25 linkage groups. Preliminary screening of an F_2 and a 4WC family identified a number of loci associated with cold tolerance and body weight. These loci were clustered in a few linkage groups, suggesting they may be indicative of quantitative trait loci.

Creating and Characterizing Genetic Variation in Tilapia Through the Creation of an Artificial Center of Origin

Objectives of Original Proposal

The overall goal of the integrated project was to develop improved breeds of tilapia by taking advantage of the ease of producing inter-specific hybrids among tilapia species and the multiple re-speciation (MRS) approach to breeding synthetic populations of tilapia tolerant to temperate climates and saline environments. The specific objectives of the research were:

1. Create an artificial center of origin (ACO) based on an inter-specific composite cross of species of tilapia.
2. Characterize the genetic variation for cold tolerance, salt tolerance and body weight in the second filial (F_2) generation, while continuing the development of the ACO by interbreeding F_1 crosses to produce four-way crosses.
3. Utilize the unique genetic material produced during the crossing program to generate a gene map of the tilapia genome using molecular genetic markers.
4. Initiate the identification of quantitative trait loci (major genes) associated with cold tolerance and salinity tolerance using molecular and quantitative genetic methods.

The project was successful in completing all of the objectives defined in the original proposal except for the assessment of salinity tolerance, which could not be completed with the available resources and funding. However, a salinity challenge protocol was developed, a initial comparative growth trial in salt- and fresh-water in progress, which will establish protocols for continuation projects directed at developing new genetic material from the ACO. The project did extend the assessment of cold tolerance performance by completing challenge tests of a four-way-cross group. The four-way cross was used for genomic mapping and screening for quantitative trait loci, in addition to the original plan of only using F_2 families for this part of the project.

Report of Results

Artificial Center of Origin (ACO)

The ACO was made using four species, including two strains of one species, for a total of five parental types: *Oreochromis niloticus* wild type (*On*), *O. niloticus* red type (*ROn*), *O. aureus* (*Oa*), *O. mossambicus* (*Om*), and *Sarotherodon galileus* (*Sg*). The mating scheme for the three four-way-cross groups making up the ACO is outlined in Appendix A, Figure 1. The *O. mossambicus* strain used for the study originated from Natal, South Africa; the *O. aureus* strain originated from Lake Hula, Israel (now drained); the wild type *O. niloticus* originated from the Volta River, Ghana; the red *O. niloticus* strain originated in Lake Manzala, Egypt and was obtained from the Institute of Aquaculture, University of Stirling, Scotland; and the *S. galilaeus* strain originated in Lake Kinneret, Israel.

Two-way crosses (F_1) were established for all six combinations of the four species and nine out of ten of the crosses involving the two *O. niloticus* strains. The F_1 groups were assigned symbolic names, with female parent listed first followed by male parent, using the abbreviated “species codes; for example *On* x *Oa* denotes a progeny group from a cross of an *O. niloticus* female with an *O. aureus* male. In addition, some of the F_1 groups were used to produce F_2 and backcross generations for genetic studies based on the performance of these groups relative to the parental types.

The three-way crosses (3WC) produced were (female parent listed first): *Om* x (*Oa* x *ROn*), *Om* x (*On* x *Oa*), *Om* x (*Sg* x *On*), and *Om* x (*Sg* x *On*). These groups were used for performance studies and for genomic mapping. Three sets of four-way crosses (4WC) were produced as the final ACO, and were used for preliminary performance tests and for genomic mapping. The 4WC groups were (with the number of families): 4WC₁, female (*Om* x *Oa*) and male (*Sg* x *On*), five families from two males and four females; 4WC₂, female (*Sg* x *Om*) and male (*On* x *Oa*), four families from one male and four females; and 4WC₃, female (*Om* x *ROn*) and male (*Sg* x *Oa*), two families from one male and two females. Each of the 4WC groups was bred *inter se* to mix the species genomes and provide material from which to extract new tilapia strains through selective breeding.

Cold Water Challenge

Tolerance to cold water was assessed using a recirculating rearing-system fitted with a temperature controller that permitted temperature reduction on a daily cycle the normal rearing temperature (about 27°C) to a low of 8°C. Preliminary trials were carried out to assess protocols for cooling and stocking of fish in the system, to evaluate the likelihood of a correlation between the size of fish and cold tolerance, and to test the concept of challenge-testing genetic groups. The parameter of ‘time to comatosis’ for each fish was recorded in degree-days measured as the accumulated temperature over the days the fish spent in the system (with day recorded to the nearest 24-h period).

The initial trial, using a single group of crossbred fish ranging in size from 30 to 70 mm, verified the reliability of the cooling system, the method of holding fish in the system, and indicated little association between fish size and cold tolerance. Trials were then undertaken to assess the ability of the system to differentiate among genetic groups, assess level of variation within and among groups, and to further assess the potential of an association between body size and cold tolerance. The second trial, using four, single-pair spawns (four full-sib groups) of *Om* determined if the challenge-test protocol could detect differences among full-sib groups and provided estimates of variation among and within families. The third trial compared four half-sib, F_2 (*Oa* x *ROn*) families from a common sire to add a half-sib comparison to the preliminary experiments. The data on cold tolerance for the second and third trials are summarized in Table 1; cumulative mortality curves are given in Figure 1.

The fourth trial was undertaken to examine performance differences among several types of genetic groups, and gain further estimates of phenotypic variances. Four of the six groups tested consisted of generational series for two parental types, *Oa* and *Om*, the F_1 [*Om* x *Oa*], and the F_2 obtained by inter-breeding the [*Om* x *Oa*] F_1 . The two additional groups were an [*Oa* x *ROn*] F_1 and its F_2 obtained by inter-mating the [*Oa* x *ROn*] F_1 . Estimates of the mean and standard deviation for cold tolerance are given in Table 2. There were significant differences among the six groups. *Oa* and the [*Om* x *Oa*] F_1 were significantly ($p < 0.0001$)

Table 1. Mean and standard deviation for degree-days of cooling for eight groups of tilapia challenged for cold tolerance to 8°C. Superscripts indicate significant differences at $p < 0.0001$.

Test Group	Degree-days	
	Mean	Std. Dev.
Fill-sib <i>Om</i>		
Om1	224.3 ^a	24.54
Om2	213.4 ^{ab}	23.37
Om3	210.5 ^b	15.57
Om4	189.9 ^c	18.50
Half-Sib <i>Oa</i> x <i>ROn</i>		
100	105.4 ^a	13.05
102	99.5 ^b	13.39
16	99.0 ^b	8.77
33	77.6 ^c	15.58

more cold tolerant than *Om* and the [*Om* x *Oa*] F₂ group. The [*Oa* x *ROn*] F₁ and its F₂ were the least cold tolerant ($p < 0.0001$) of all the groups.

The correlation between standard length and cold tolerance was very low ($r^2 = 0.041$, 0.023, 0.029 for three replicates) and there was no correlation between body color and cold tolerance ($r^2=0.004$, $p = 0.268$). Thus, it appeared fish size did not influence time in degree-days from exposure to death. Significant differences were detected among families at both the full-sib and half-sib level. The standard deviation was higher within full-sib groups than half-sib groups, but this may have been due to variation among trials and fish with different species origins.

Figure 1. Cumulative mortality curves from cold-challenge tests of four, full-sib *O. mossambicus* groups.

Table 2: Mean and standard deviation (Std Dev) for cold tolerance (Degree-Days) for six genetic groups, listed in descending order of tolerance. Superscripts indicate significant differences at $p < 0.0001$.

Strain	Degree Days	
	Mean	Std Dev
<i>Oa</i>	235.6 ^a	22.39
$F_1(Om \times Oa)$	230.3 ^a	20.15
<i>Om</i>	175.9 ^b	20.15
$F_2(Om \times Oa)$	166.5 ^b	25.89
$F_2(Oa \times ROn)$	100.7 ^c	22.79
$F_1(Oa \times ROn)$	95.1 ^c	23.13

Based on the preliminary results, it was concluded that the cold-challenge system gave reliable data, the protocol was capable of detecting differences among genetic groups, and no significant relationship existed between body size and cold tolerance at the body sizes to be used for initial cold tolerance studies. However, it was decided that the parameter used to measure cold tolerance could be improved by not considering the period of cooling required to reduce the temperature from normal rearing temperature to 16°C. Using the full-time period placed excess emphasis on the early days of the trials, as all fish were able to survive through cooling to 16°C.

A new definition for the cold-tolerance parameter was designed to place emphasize on an individual's ability to survive through the lower temperatures. The new parameter, termed "Cooling-Degree Days" (CDD), was defined for each fish, as the difference between the daily temperature and 16°C, summed over all days from the day the cooling system reach 16°C to the day the fish became comatose. The parameter is defined as:

$$CDD = \sum_{i=1}^k (t_0 - t_i) \quad (1)$$

where i = days, t_0 = 16°C, the initial temperature, t_i = temperature at the i^{th} day, and k = day of mortality. For example, the score for a fish that survived 1 day in 15.5°C and 1 day in 15°C, is $(16 - 15.5) + (16 - 15) = 1.5$ CDD.

To begin estimating genetic effects for cold tolerance, replicate cold-challenge tests were completed using a three-generation series consisting of two parental types, *Om* and *Oa*, and their F_1 and F_2 crosses. Two 4WC crosses were added to the second replicate, 4WC₁ [(*Om* x *Oa*) x (*Sg* x *On*)] and 4WC₂, [(*Sg* x *Om*) x (*On* x *Oa*)]. Data from the second replicate was used for initial estimation of QTL effects with the F_2 family and 4WC₁. The data are summarized in Table 3 and cumulative mortality curves are presented in Figures 2 and 3.

Table 3. Mean and coefficient of variation (CV) for cold tolerance (cooling-degree days, CDD) for *O. aureus* (*Oa*), *O. mossambicus* (*Om*) and their F₁ and F₂ crosses, plus two 4-way crosses (4WC1 and 4WC2, as defined in the text). Superscripts indicate significant differences at $p < 0.01$.

Genetic Stock	Replicate 1			Replicate 2		
	N	Mean CDD	CV (%)	N	Mean CDD	CV (%)
<i>Oa</i>	54	98.5 ^a	17.3	58	88.1 ^a	11.7
F ₁	57	98.2 ^a	14.6	58	87.7 ^a	14.6
F ₂	55	83.3 ^b	23.6	59	75.9 ^b	13.8
<i>Om</i>	56	85.4 ^b	17.0	42	49.7 ^d	25.8
4WC ₁				47	67.2 ^c	13.1
4WC ₂				57	65.5 ^c	15.4

The response of the parental types and their crosses was consistent over the two replicates, except for the low for the *Om* parental type in Replicate 2. Across replicates, the F₁ hybrid did not differ in cold tolerance from the *Oa* parental type and both *Oa* and the F₁ were more cold tolerant than the F₂ group. This ranking of generational groups suggests dominance for *Oa* cold-tolerance allele(s). As a result of the low response of the *Om* parental type in Replicate 2, the differences between the mid-parent value and the F₁ were +6.25 and +18.5 CDD for replicates 1 and 2, respectively; the same differences were -8.65 and +7.00 CDD for the F₂ groups.

If complete dominance is assumed for the *Oa* allele(s), then the F₂ is expected to segregate in a ratio of 3 *Oa* to 1 *Om*. Using the appropriately weighted parental values as an expectation for the F₂, the deviations from expectation were negative (-11.9 and - 2.6 CDD) in Replicates 1 and 2, respectively, a reasonable fit to expectation for these relatively small sample sizes. The estimated cold tolerance of the two 4WCs included in Replicate 2 was lower than that for all genetic groups except the *Om* parental type. However, since only two of the four parental types contributing to the 4WCs were included in the trial, no interpretation can be made from this observed response.

Figure 2. Cumulative mortality curves in cold-challenged full-sib groups of *O. aureus*, (---), *O. mossambicus* (— —) and their F₁ (—) and F₂ (— — —) hybrids

Figure 3. Cumulative mortality curves in cold-challenged full-sib groups of *O. aureus* (---), *O. mossambicus* (— —), their F₁ (—) and F₂ (— — —) hybrids, and two 4WC hybrids (solid and dashed light lines).

Genome Mapping

The original plan for the project was to use RAPD technology to obtain a large number of molecular markers in a short period of time. However, UCDavis laboratory changed directions and adopted the use of AFLP (Amplified Fragment Length Polymorphism) markers as they do not suffer from the reproducibility problems associated with RAPD markers. The first attempt to adapt the AFLP technology to tilapia was done using a single, six-base-cutting, restriction enzyme (Pst I) to digest the genome, and then to visualize the amplifications on Metaphor agarose gels stained with ethidium bromide. The amplifications worked as expected and the method revealed inter-specific variation for all the species involved in the ACO. However, the Metaphor agarose gel did not give the resolution necessary to detect a large number of loci.

The laboratory acquired a laser-based, fluorescent, gel-scanning system (Molecular Dynamics' Fluorimager 595) capable of scanning polyacrylamide gels. Separation with polyacrylamide improved resolving power greatly, but the single-enzyme AFLP method still limited the number polymorphic loci that could be detected. Consequently, effort was directed toward the development of a two enzyme-digestion AFLP method, the approach most often used for mapping studies. The method adopted was the use of a frequent-cutter enzyme (MseI) in combination with an infrequent cutter (EcoRI) and to varying the primer sequences for primers designed for each of the restriction fragments to obtain an array of possible primer combinations.

Table 4 shows the primers used for the development work; the list contains only a small number of the total Mse primers available for a single Eco primer. The range of fragment sizes obtained with the Pst I based method was from 0.5 kbp to 2 kbp. The Eco-Mse based method reduced fragment sizes to a more desirable range of 25 bp to 500 bp. These fragment sizes made identification of loci highly repeatable from run to run, and along with the fluorescent gel-scanner technology, greatly simplified the process of elucidating markers for genomic mapping. A sample of six individuals from each of the five strains used to develop the ACO was used to establish the viability of the marker protocols.

Genomic mapping was initiated with a 3WC family obtained by crossing an *Om* female to an (*Oa* x *ROn*) F₁ male. The DNA from the parents of the 3WC and the grandparents of the F₁ male was analyzed along with 66 individuals from the 3WC. To provide species comparisons, DNA from six individuals of each of the five strains used to make the ACO was run along with the mapping panel. Joint segregation of *Oa*- and *ROn*-specific markers were detectable, and *Om* specific bands provide a background that did not interfere with visualization of the *Oa*-specific and *ROn*-specific bands. The 133 microsatellite primers developed for *O. niloticus* by Lee and Kocher (Microsatellite DNA markers for genetic mapping in *Oreochromis*, J Fish Biol. 49:169-171, 1996) also were used in the mapping study.

Table 4. Adapter oligos and primers evaluated for genomic mapping. "F" indicates a primer labeled with Fluorescein.

Oligo	Sequence (5'-3')	Use
EcoA1	CTC GTA GAC TGC GTA CC	adapter
EcoA2	AAT TGG TAC GCA GTC	adapter
MseA1	GAC GAT GAG TCC TGA G	adapter
MseA2	TAC TCA GGA CTC AT	adapter
EcoG	GAC TGC GTA CCA ATT CG	Pre-Amp Primer
EcoGCT(F)	F-GAC TGC GTA CCA ATT CGC T	Selective primer
MseC	GAT GAG TCC TGA CTA AC	Pre-Amp Primer
MseCG	GAT GAG TCC TGA CTA ACG	Selective primer
MseCGT	GAT GAG TCC TGA CTA ACG T	ditto
MseCGTC	GAT GAG TCC TGA CTA ACG TC	ditto
MseCA	GAT GAG TCC TGA CTA ACA	ditto
MseCT	GAT GAG TCC TGA CTA ACT	ditto
MseCC	GAT GAG TCC TGA CTA ACC	ditto
MseCGC	GAT GAG TCC TGA CTA ACG C	ditto
MseCAG	GAT GAG TCC TGA CTA ACA G	ditto
MseCAT	GAT GAG TCC TGA CTA ACA T	ditto
MseCTG	GAT GAG TCC TGA CTA ACT G	ditto
MseCTA	GAT GAG TCC TGA CTA ACT A	ditto
MseCCG	GAT GAG TCC TGA CTA ACC G	ditto
MseCCT	GAT GAG TCC TGA CTA ACC T	ditto

A total of 330 AFLP loci were segregating in the 3WC mapping family, 63 from the *Om* female parent and 267 from the F₁ male parent. The AFLP primer combinations and the

numbers of polymorphic loci amplified for each combination are listed in Table 5. The female (*Om*) parent had a total of 78 markers segregating (17 microsatellite, 61 AFLP). Of these, 62 (13 microsatellite, 49 AFLP) were resolved into 14 linkage groups covering a total of 514 cM (Appendix A: Figure 2). The F₁ hybrid (*Oa* x *ROn*) male had a total of 229 markers segregating (62 microsatellite, 167 AFLP) and 214 (60 microsatellite, 154 AFLP) were mapped into 24 linkage groups covering a total of 1632 cM (Appendix A: Figure 3).

Table 5. The 30 primer combinations used to generate the AFLP Markers and the numbers of polymorphic markers identified for the parent of the 3WC.

Enzyme and extender		Number variable loci 3WC parents			Enzyme and extender		Number variable loci 3WC parents	
Eco	Mse	Female	Male		Eco	Mse	Female	Male
GA	GAG	3	8		GG	CAT	1	5
GA	CGA	1	4		GG	CCC	3	5
GA	CGC	5	9		GG	CCT	0	8
GA	CGG	2	3		GG	CGC	2	2
GA	CTA	0	6		GG	CGG	2	1
GA	CTC	3	4		GG	CGT	2	4
GA	CTG	1	11		GG	CTA	4	6
GA	CTT	1	6		GG	CTC	2	8
					GG	CTT	3	5
GC	CAA	4	8		GT	CCT	1	7
GC	CAT	1	3		GT	CGC	0	3
GC	CCC	1	1		GT	CGG	3	3
GC	CCG	1	3		GT	CTA	2	8
GC	CCT	3	5		GT	CTC	0	8
GC	CTG	2	7		GT	CTG	3	8
					GT	CTT	4	8

Species-specific markers were identified for the four species used to produce the synthetic stock. Analysis of the distribution of 608 AFLP markers scores, 203 were species specific (33%) with 92 markers specific to *Sg*, 64 to *Om*, 24 to *Oa*, and 23 specific to *On*.

Two additional genomic maps were produced using a 4WC₁ family. Two linkage maps, referred to as female and male linkage maps, were created from segregation within each F₁ parent, (*Om* x *Oa*) female and (*Sg* x *On*) male. To simplify the development of the maps, polymorphic AFLP markers were identified independently for each parent to identify segregating loci. Consequently, AFLP components of linkage groups on the two maps cannot be compared directly; however, they can be aligned using the microsatellite loci common to both the female and male maps.

The female linkage map contained 131 markers (39 microsatellite, 92 AFLP) resolved into 26 linkage groups covering a total of 806.1 cM (Appendix A: Figure 4). The male linkage map contained 128 markers (36 microsatellite, 82 AFLP) resolved into 25 linkage groups covering a total of 843.5 cM (Appendix A: Figure 5). The female (*Om* x *Oa*) linkage groups are labeled MxA_1 through MxA_26, and the male (*Sg* x *On*) linkage groups are labeled GxN_1" through GxN_25, to distinguish them from linkage groups reported from other studies.

A partial alignment of the four genomic maps was achieved based on microsatellite loci showing variation in all maps. The alignment is listed in Appendix A: Table 1. Further alignment of the maps will be possible when the microsatellite loci currently under development are available for general use by the scientific community.

Identification of Putative QTL

The marker systems developed during the genomic mapping studies were used for an initial search for quantitative trait loci (QTL) associated with cold tolerance and body weight. The initial trial was carried out with 60 fish from an F₂ (*Oa* x *Om*) family (Replicate 2, Table 2), using 20 microsatellite markers. Two alleles were present at all the loci except UNH159,

which had three alleles. Loci showing an association with cold tolerance and body weight are listed in Table 6, along with the mean performance of each marker genotype.

A statistically significant association was found between two microsatellite markers, UNH180 ($p=0.0102$) and UNH118 ($p=0.0119$) and cold tolerance. For both markers, individuals homozygous for the slow-migrating band (SS) were 9% to 10% more tolerant than the overall average (75.9 g), while those homozygous for the fast band (FF) were 6% to 7% less tolerant than the average. Three markers, UNH197 ($p=0.0042$), UNH180 ($p=0.0271$) and UNH183 ($p=0.0283$), were found to be associated with body weight. For the two genotypes at UNH197, the average weight of the homozygous individuals was higher than that of heterozygotes. Individuals homozygous for the fast UNH180 band were 22% heavier than the average (10.4 g) and those homozygous for the slow band were 30% lighter than the average, while the heterozygotes appeared to be intermediate in body weight.

Table 6. Association of microsatellite marker genotypes (SS, FS and FF) with cold tolerance (cooling degree day, CDD) and body weight (g) in an F_2 family. Column list marker number (Kocher et al., 1998), the probability (P) of the F-statistic (F), the R^2 for the association, mean for each marker genotype along with percent deviation from the F_2 average (75.9 ± 10.5 CDD, and 10.4 ± 5.9 g). For each marker, different superscripts indicate significant differences ($p < 0.05$) among genotypes.

Marker	N	P	F	R ²	Microsatellite Genotype		
					SS	FS	FF
Cold Tolerance (CDD)							
180	55	0.0102	5.01	0.16	82.6 (+9%) ^a	76.7 (+1%) ^{ab}	71.3 (-6%) ^b
118	51	0.0119	4.87	0.17	83.8 (+10%) ^a	75.1 (-1%) ^b	70.8 (-7%) ^b
159	55	0.0784	2.24	0.15			
Body Weight (g)							
197	56	0.0042	8.95	0.14	8.6 (-17%) ^a	13.2 (+26%) ^b	-
180	56	0.0271	3.86	0.13	7.3 (-30%) ^b	9.9 (-5%) ^{ab}	12.7 (+22%) ^a
183	56	0.0283	3.81	0.13	6.5 (-38%) ^b	11.4 (+10%) ^a	6.7 (-38%) ^b
138	59	0.0770	2.69	0.09			
231	57	0.0810	2.63	0.09			

For UNH183, the average weight of the heterozygous individuals was higher than that of both homozygotes. The correspondence between average performance of the homozygous F_2 genotypes and the *Oa* and *Om* parental types suggest relatively tight linkages.

The second experiment to identify putative QTL was undertaken with 54 fish from the 4WC₁ [(*Om* x *Oa*) female and (*Sg* x *On*) male]. The 4WC₁ fish were screened with AFLP and microsatellite markers and examined for association with observed cold tolerance (CDD) and body weight (g). A QTL analysis was done separately for marker data for each of the F₁ parents. Since each parental type could contribute two alleles to their progeny, the marker genotypes of the fish were classified as having the allele (h: band present) versus not having the allele (a: band absent for AFLP loci; alternate allele for microsatellite loci). The mean performance of 4WC₁ was 67.2 ± 1.39 CDD for cold tolerance and 7.92 ± 0.51 g for body weight (Table 3). The minimum observed cold tolerance (42.5 CDD) corresponded to a fish surviving to 10.5°C, which took 11 days, while the maximum (88.5 CDD) corresponded to a fish surviving to a temperature of 9°C, which took 18 days.

Three AFLP loci and one microsatellite locus from the female and six AFLP loci and three microsatellite loci from the male, showed positive association with cold tolerance of the 4WC₁ progeny (Table 7). The markers from the female map were located in two linkage groups, while those from the male were located in four linkage groups. Genotypes at two AFLP loci and one microsatellite locus from the female, and four AFLP loci and three Microsatellite loci from the male were associated with differences in body weight (Table 8). Six of the seven markers for the male were located in two linkage groups.

It was impossible to distinguish between tight linkage to a QTL of small effect and loose linkage to a QTL of large effect when studying QTL one marker at a time. Also, some of the putative linkages reported here cannot be considered statistically significant in the context of a whole genome scan with multiple tests. Markers with probabilities of association of less than 0.01 are reported only when they were genetically linked to other marker(s) with higher significance values. For example, AFLP marker “GAACTA129” (Table 8, $p = 0.0294$) and the microsatellite marker “UNH130” ($p = 0.0142$) exhibited a loose association with body weight, but would not be reported except for the fact that they are linked to the AFLP “GCACAC060” ($p = 0.0003$).

The species of origin (*Om* or *Oa*) of each allele for locus polymorphic in the F₁ female was determined from the genotypes of the maternal grandparent. In every case, the allele

originating from the *Om* grandparent was associated with superior body weight and cold tolerance. Data was not available for the paternal grandparents.

Table 7. Marker loci exhibiting association of cold tolerance (CDD) from female-informative and male-informative loci, the linkage group for the marker, the probability the association is zero (P), and phenotypic means and standard error for the genotype with the allele (h) and without the allele (a = band absent for AFLP loci, alternate allele for microsatellite loci).

Marker Name	Linkage Group	P	Genotype “h” (mean $\pm$ se)	Genotype “a” (mean $\pm$ se)
Female map				
GAACAT158	MxA-2	0.0127	70.20 $\pm$ 2.18	62.29 $\pm$ 2.13
GAACTA118	MxA-2	0.0395	62.59 $\pm$ 1.97	68.66 $\pm$ 2.09
GAACCG117	MxA-14	0.0206	62.45 $\pm$ 1.91	70.37 $\pm$ 2.70
UNH169	MxA-14	0.0096	70.68 $\pm$ 2.37	62.50 $\pm$ 1.89
Male map				
GAACAT289	GxN-3	0.0374	68.05 $\pm$ 1.99	60.80 $\pm$ 2.72
GCACCC706	GxN-3	0.0022	59.18 $\pm$ 2.30	68.68 $\pm$ 1.83
UNH179	GxN-3	0.0025	60.67 $\pm$ 2.03	69.41 $\pm$ 1.85
GAACTA103	GxN-23	0.0109	62.54 $\pm$ 1.73	70.24 $\pm$ 2.34
UNH123	GxN-23	0.0029	62.21 $\pm$ 1.75	71.71 $\pm$ 2.47
GAACAT049	GxN-10	0.0405	68.74 $\pm$ 2.27	62.04 $\pm$ 2.22
GCACAT067	GxN-10	0.0071	68.98 $\pm$ 1.92	61.23 $\pm$ 1.99
UNH203	GxN-12	0.0431	66.48 $\pm$ 1.76	60.55 $\pm$ 2.25
GAACAA109	GxN-12	0.0091	68.69 $\pm$ 1.91	61.00 $\pm$ 2.10

The 4WC₁ individuals used in the search for putative QTL are non-traditional in that the parental stocks were not inbred, and in fact, should be assumed to be highly heterozygous. If each parent is heterozygous for unique alleles for both marker loci and loci affecting the quantitative trait, then up to four different alleles could be segregating at each locus. All alleles are visible for codominant microsatellite loci. However, dominance of alleles at AFLP loci complicates the interpretation of genotypes recorded as band present or absent, and discrimination can be achieved for only two of the possible four alleles at a locus. The QTL analyses assumed that all “null” AFLP genotypes (“a” genotype in Tables 7 and 8) were homozygous for the same allele. This was most likely not true in many cases since the parents were hybrids of different species; the scoring system actually “binned” all of the “null” alleles

into one. This “binning” of alleles will result in a tendency to underestimate differences between the genotypic classes, reducing detection of QTL. Therefore, the associations reported from this study can be considered conservative since the method most likely underestimated the true associations between marker loci and the underlying QTL.

Table 8. Marker loci exhibiting association of body weight (g) from female-informative and male-informative loci (columns as in table 7).

Marker	Linkage Group	p	Genotype “h” (g ± se)	Genotype “a” (g ± se)
Female map				
GAACCG358	MxA-20	0.0099	9.04 ± 0.66	6.20 ± 0.83
GCACAT410	MxA-10	0.0148	6.64 ± 0.61	9.12 ± 0.76
UNH104	Unlinked	0.0134	6.76 ± 0.65	9.30 ± 0.74
Male map				
UNH124	GxN-1	0.0095	6.68 ± 0.67	9.42 ± 0.76
GAACTA129	GxN-2	0.0294	9.14 ± 0.72	6.86 ± 0.72
GCACAC060	GxN-2	0.0003	9.90 ± 0.66	6.20 ± 0.68
UNH130	GxN-2	0.0142	9.25 ± 0.71	6.77 ± 0.66
GAACTA098	GxN-8	0.0053	8.70 ± 0.54	5.23 ± 1.06
GAACCT148	GxN-8	0.0289	8.60 ± 0.61	5.88 ± 1.03
UNH138	GxN-8	0.0266	8.78 ± 0.69	6.52 ± 0.71

Cooperation

The project was collaborative in every respect. The parental stocks (the species of tilapia) used to produce the ACO were available in pure form only at the ARO (Israel) station, as were the facilities essential for the breeding program. Thus, the opportunity to create the ACO provided a unique opportunity for the integration of a multi-disciplinary project with complementary objectives: the development of new breeds of tilapia, application of quantitative genetics to breed development, genetic mapping of the genome, and identification of associated QTL for economically important performance traits. In addition, the project was the first of its kind involving species crosses as an approach to breed development of an aquacultural species of fish.

The three institutions shared responsibilities for the project. All institutions participated in the design and development of experiments and the analysis of results. The breeding and management of the fish populations and the evaluation of fish performance was carried out by G. Hulata at the Department of Aquaculture, Agricultural Research Organization, Volcani Center, Bet Dagan, Israel (ARO station) with assistance from N. Umiel.

Initial screening of species for molecular markers and the production of the genetic linkage map were done by G. Gall and B. May, Department of Animal Science, University of California, Davis, USA (UCD station) and E. Hallerman, Department of Fisheries & Wildlife Sciences, Virginia Polytechnic Institute and State University, Blacksburg, USA (VPI station). The final effort to define a genomic map was completed by G. Gall and B. May at the UCDavis station, using both three-way-cross and four-way-cross genetic material. Detection of QTL associated with cold tolerance and body weight was initiated by the VPI station using an F₂ generation produced early in the project. The majority of the work on linkage analysis was done by the UCD station because of the availability of equipment, resources and expertise.

The distribution of expertise among the collaborators was a major contributor to the success of the project. The culture of tilapia, and the evaluation of breed potential, has been part of a long-standing program at the ARO station. The UCD station has extensive expertise in the application of molecular marker technology, genetic marker development, the study of genetic diversity, and the application of quantitative genetics to fish breed improvement.

Evaluation of Research Achievements

Preliminary examination of composite crosses that are part of the ACO demonstrated the existence of large variation among phenotypes that will provide unusual material for a breed improvement program for tilapia, and subsequently, the construction of more detailed genomic maps. Analysis of the unique genetic material also provided evidence of QTL for at least one trait of great importance to future food production from tilapia, variation in tolerance to cold water. This information will be of direct benefit to the long-term project goal of developing new stocks of tilapia.

Performance evaluations of species included in the ACO confirmed published results that *O. mossambicus* is more cold sensitive than *O. aureus*. Differences among the pure species and their crosses indicated a large dominance genetic component; this result was suggested earlier by Wohlfarth et al. (Comparisons between interspecific tilapia hybrids for some production traits. In: International Symposium on Tilapia in Aquaculture, L. Fishelson and Z. Yaron (eds), Tel Aviv University, 1983). The higher cold tolerance observed in the first replicate of the cold-water challenge experiment, compared to the second, may reflect a difference due to season of testing; the first replicate was carried out in April, while the second was carried out in August. It is possible fish that have recently experienced winter temperatures are better acclimated to low temperature than those grown at a higher temperature prior to testing.

Since meiosis in two parents are independent events, the use of a three-way cross family for genomic mapping resulted in two separate data sets made it possible to define two linkage maps, one for the two species contributing to the F₁ parent plus one for the pure-species parent. Similarly, two maps resulted from screening a four-way cross, each map representing linkage among markers segregating in each F₁ parent. These linkage maps could be aligned using the microsatellite markers common to all maps.

Tilapia culture in temperate regions is affected negatively by the species' sensitivity to low temperature, so the identification of QTL for cold tolerance should enhance a breeding program designed to create cold tolerant strains. The project was successful in identifying a number of putative QTL. Screening an F₂ family (*O. aureus* x *O. mossambicus*) identified two markers (UNH118 and UNH180) associated with cold tolerance and three markers (UNH180, UNH183 and UNH197) associated with body weight. Two of those markers, UNH180 and UNH197, were located in the same linkage group, increasing the likelihood the association is real. Screening a four-way-cross family [(*O. mossambicus* x *O. aureus*) female x (*S. galilaeus* x *O. niloticus*) male with AFLP and microsatellite markers identified ten markers associated with cold tolerance, representing only 6 of a possible 22 chromosomal regions and thirteen markers associated with body weight, representing only 5 chromosomal regions. None of the AFLP or microsatellite markers attain the strict guideline of $p < 10^{-4}$ suggested for "significant linkage" to a QTL, but the results argue that many of the regions identified contain several markers associated with one or the other of the two quantitative traits.

Conclusions

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 (*ROn*) strains], *O. aureus* (*Oa*), *O. mossambicus* (*Om*), and *Sarotherodon galilaeus* (*Sg*). All possible two-way crosses (F_1), a number of three-way crosses (3WC) and three four-way crosses (4WC) were produced to establish the ACO and provide genetic material for studies on the genomic map of tilapia. The three 4WC groups contain equal representation of all four species, and have been retained as the ACO.

Four genomic maps were created using microsatellite and AFLP markers, two from a 3WC family (*Om* female and (*Oa* x *ROn*) male) and two from a 4WC family [(*Om* x *Oa*) females and (*Sg* x *On*) male]. The female (*Om*) parent of the 3WC had a total of 78 segregating markers (17 microsatellite, 61 AFLP). Of these, 62 (13 microsatellite, 49 AFLP) were placed in 14 linkage groups covering a total of 514 cM. The F_1 male parent of the 3WC had a total of 229 segregating markers (62 microsatellite, 167 AFLP), of which 214 (60 microsatellite, 154 AFLP) were placed in 24 linkage groups covering a total of 1632 cM. A linkage map, based on the female F_1 parent of the 4WC, contained 131 markers (39 microsatellite, 92 AFLP) that resolved into 26 linkage groups covering a total of 806.1 cM; mean inter-marker distance was 6.15 cM. The linkage map derived from the F_1 male parent of the 4WC contained 128 markers (36 microsatellite, 82 AFLP) that resolved into 25 linkage groups covering a total of 843.5 cM with a mean inter-marker distance of 6.6 cM. The maps were aligned using common microsatellite markers.

Screening of an F_2 family (*Oa* x *Om*) with microsatellite markers identified two markers with putative association with cold tolerance and three associated with body weight. Two of the markers were located in the same linkage group, increasing the likelihood the association is real. Screening of an 4WC family with both microsatellite and AFLP markers identified ten markers with likely association with cold tolerance; the markers were located in

only 6 chromosomal regions. Thirteen markers with apparent association with body weight were located in only 5 chromosomal regions.

List of Publications

- Agresti, J.J., S. Seki, A. Cnaani, S. Poompuang, E.M. Hallerman, N. Umiel, G. Hulata, G.A.E. Gall, and B. May. 2000. Breeding new strains of tilapia: development of an artificial center of origin and linkage map based on AFLP and microsatellite loci. *Aquaculture* 184:(In press).
- Cnaani, A., G.A.E. Gall and G. Hulata. 2000. Cold tolerance of tilapia species and hybrids. *Aquaculture International* (In press).
- Agresti, J.J., A. Cnaani, T.R. Famula, G. Hulata, G.A.E. Gall, and B. May. Additional genomic map information and associations of molecular markers with cold tolerance and body weight from a hybridized stock of tilapia. (In preparation).
- Cnaani, A., G. Hulata, G.A.E. Gall, M. Indelman, Y. Kashi, and E.M. Hallerman. Detection of putative QTLs for body weight and cold tolerance in interspecific tilapia hybrids. (In preparation)
- Seki, S. J.J Agresti, G.A.E. Gall, N. Taniguchi and B. May. 1999. AFLP analysis of diversity and status of three populations of ayu (*Plecoglossus altivelis*). *Fisheries Science* 65:888-892
- Palti, Y., S. Tinman, A. Cnaani, Y. Avidar, M. Ron, and G. Hulata. 2000. Characterization of immunological and biochemical differences between two tilapia species (*Oreochromis aureus* and *O. mossambicus*). *The Israeli Journal of Aquaculture – Bamidgeh* (In press).
- Palti, Y., A. Shirak, G. Hulata, M. Ron, and R.R. Avtalion. Detection of genes with deleterious alleles in an inbred line of tilapia (*Oreochromis aureus*). (In preparation).
- Palti, Y., A. Shirak, A. Cnaani, E. Feldmesser, R.R. Avtalion, G. Hulata, and M. Ron. A microsatellite marker harbors a limited number of copies in the genome of two tilapia species (*Oreochromis aureus* and *O. niloticus*). (In preparation).

Appendix A

Appendix A: Table 1.

Alignment of linkage groups across five tilapia genomic maps arranged by microsatellite loci (Kocher, et al. 1998. A genetic linkage map of the Cichlid fish, the Tilapia (*Oreochromis niloticus*). Genetics 148, 1225-1232).

Appendix A: Figure 1.

Diagram of the mating scheme used to produce the four-way crosses defined as the Artificial Center of Origin. The symbols used to represent to pure-species strains: O = *Oreochromis*, S = *Sarotherodon*, a = *aureus*, m = *mossambicus* n = *niloticus*, g = *galilaeus*, and R = red phenotype.

Appendix A: Figure 2.

A linkage map prepared from the female (*Om*) parent of the 3WC mapping family. Microsatellite loci are identified by “UNH” and a 3-digit identification number, and emphasized in bold text. AFLP loci are identified by G*C**NNN, where “G*” refer to the Eco selective nucleotides, and the “C**” refers to the Mse selective nucleotides.

Appendix A: Figure 3.

A linkage map prepared from the male (*ROn x Oa*) parent of the 3WC mapping family. Microsatellite loci are identified by “UNH” and a 3-digit identification number, and are emphasized in bold text. AFLP loci are identified by G*C**NNN, where “G*” refer to the Eco selective nucleotides, and the “C**” refers to the Mse selective nucleotides. The linkage groups are numbered with the prefix “UCD” to differentiate them from maps from other studies.

Appendix A: Figure 4.

AFLP and microsatellite linkage map from the (*O. mossambicus* x *O. aureus*) female used to produce 4WC₁. Cumulative linkage distances in Kosambi cM are to the left of the linkage group and locus names are on the right. Microsatellite loci are in bold and named “UNHXYZ” from Kocher *et al.* (1998). All other loci listed represent those corresponding to AFLP markers.

Appendix A: Figure 5.

AFLP and microsatellite linkage map developed from the (*S. galilaeus* x *O. niloticus*) male used to produce 4WC₁. Linkage distances and locus nomenclature is the same as in Figure 4.

Appendix Table 1. Alignment of linkage groups across five tilapia genomic maps arranged by microsatellite loci (Locus). Columns GN and MA are the male and female linkage groups from the 4WC study; columns AR and M are the male and female linkage groups from the 3WC study; column N is linkage group from Kocher *et al.* (1998).

Locus	GN	MA	AR	M	N	Locus	GN	MA	AR	M	N
102	-	19	3	-	16	168	-	-	11	-	3
103	-	12	UL	UL	17	169	7	14	5	-	10
104	12	UL	-	-	1	170	-	-	13	-	-
106	-	13	UL	UL	14	172	-	6	13	-	4
108	24	24	6	-	2	173	-	-	1	-	13
110	14	16	-	-	-	174	-	-	4	1	20
111	-	-	13	-	7	176	-	-	7	-	-
115	6	-	11	-	3	178	-	-	1	2	9
116	-	25	-	-	-	179	3	-	-	-	7
117	7	-	-	-	-	180	-	21	10	-	-
118	-	-	14	-	-	184	-	3	-	-	-
120	-	-	-	-	18	185	UL	-	20	-	-
123	23	9	19	8	12	186	-	-	-	-	15
124	1	7	13	3	-	187	21	4	8	-	-
125	8	26	3	-	16	188	-	-	1	5	-
127	-	-	-	-	13	189	23	9	19	8	12
129	13	10	2	-	8	190	9	22	9	-	21
130	2	-	10	-	23	192	19	3	7	-	11
131	6	25	-	-	3	193	-	-	UL	-	1
132	11	23	1	2	9	194	-	-	-	-	22
134	UL	-	-	-	-	196	15	-	16	-	-
135	-	-	-	-	3	197	-	5	10	-	23
137	19	9	-	-	-	201	-	-	-	-	4
138	8	26	3	-	16	203	12	6	16	UL	-
140	24	24	6	-	2	204	14	16	18	13	-
141	5	11	5	9	-	207	-	-	12	-	-
142	21	1	-	-	-	208	-	-	21	-	-
144	-	2	-	-	-	209	20	-	4	-	-
145	13	10	-	-	8	210	-	-	6	-	-
146	1	7	13	-	4	211	-	4	20	-	30
147	-	UL	1	2	9	212	-	-	22	1	15
148	12	UL	23	-	-	213	-	-	23	-	1
149	-	-	-	9	5	214	-	-	5	-	10
151	-	-	-	-	5	216	-	-	10	-	23
153	-	-	9	-	-	217	-	-	15	-	-
154	-	-	-	-	6	219	-	-	7	-	11
155	18	15	-	-	-	222	24	24	6	-	2
159	24	24	-	-	2	223	-	-	-	-	3
160	13	-	12	UL	6	226	-	-	7	7	-
161	1	7	-	-	-	227	-	-	2	-	-
162	-	-	24	-	19	228	24	24	6	-	-
163	-	6	16	-	-	231	-	-	12	-	6
165	-	-	2	-	-	232	-	-	7	-	11
166	-	23	1	-	9	233	-	-	15	UL	-

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