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A deleterious effect associated with UNH159 is attenuated in twin embryos of an inbred line of blue tilapia *Oreochromis aureus*

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Offspring of a highly inbred gynogenetic line of *Oreochromis aureus* displayed 12-fold increase in twinning rate compared to the outbred population. Asymmetric conjoined twins, which consist of a normal embryo attached to a malformed-atrophic twin, were frequently encountered in both gynogenetic (90·7%) and outbred (38·2%) embryos. The monozygotic origin of these twins was determined using five microsatellite markers. Progeny of heterozygous parents for the microsatellite UNH159 were separated into sub-sets of twins and normal full-sibs. Consistent with previous reports, the normal embryo sub-set exhibited elimination of both types of homozygotes for the UNH159 genetic marker at 2–8 days after fertilization. Unexpectedly, this elimination was less frequent in twins. The UNH159 marker as well as RNA-binding motif protein, X-linked (*rbmx*), SRY-box containing gene 3 (*sox3*) and alpha-thalassemia/mental retardation syndrome X-linked (*atrx*) genes were mapped to linkage group 2. These gene orthologues are all located on the mammalian X chromosome and *atrx* is necessary for the X-chromosome inactivation. © 2012 The Authors
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Key words: gynogenetic line; microsatellite marker; monozygotic; twinning.

INTRODUCTION

The spontaneous occurrence of conjoined twins has been reported in many bony fish species, including salmonids (Yamamoto *et al*., 1996), poecilids (Reichenbach-Klinke, 1972) and cyprinids (Gomelsky, 2003). Incidences of conjoined twins in *Oreochromis* species (Cichlidae) have also been reported in pure-bred blue tilapia *Oreochromis aureus* (Steindachner 1864), *Oreochromis mossambicus* (Peters 1852), *Oreochromis niloticus* (L. 1758) and in interspecific tilapia hybrids (Hulata & Rothbard, 1978; Huang *et al*., 1987). All twin types have a common yolk sac and in many cases they are conjoined by other body parts. The development of twins can

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be easily followed during the developmental period from hatching to complete yolk-sac resorption. Frequently, one of the two conjoined twins appears with a deformed body closely after hatching (Holden & Bruton, 1993). Conjoined twins were experimentally induced by chemical or high-temperature treatments in various fish species [*e.g. Danio rerio* (Hamilton 1822); Ingalls *et al*., 1969: *O. aureus* and *O. niloticus*; Owusu-Frimpong & Hargreaves, 2000].

The majority of induced mammalian conjoined twins were monozygous of the monochorionic–monoamniotic type (O'Shea & Kaufman, 1978; Kaufman, 1982). This type, however, is rare among spontaneous mammalian twins (Bulmer, 1970; Wharton *et al*., 1968). Dizygous twinning and family history of twinning indicating genetic predisposition are more common in mammals (Kaufman, 2004). In contrast, and with very few exceptions, this does not appear to be the case in mammalian monozygous twinning (Harvey *et al*., 1977; Shapiro *et al*., 1978). No published data on genetic analysis of twin origin (monozygous or dizygous) in fishes could be found in the literature. Palti *et al*. (2002) reported genetic markers that were associated with deleterious effects in a meiogynogenetic tilapia line (BIU-1). In this gynogenetic line, the number of polymorphic microsatellite markers gradually decreased from 76 in the original stock to 20, 11 and seven in the third, fourth and fifth generations, respectively (Palti *et al*., 2002; Shirak *et al*., 2006). By examining dozens of morphological and immunological traits for variability in this line, high uniformity was observed. The trait of cytotoxic reaction of blood leucocytes, however, was still variable in the fifth generation and associated with two of the microsatellite markers (Shirak *et al*., 2006). Preliminary observations indicted that twinning rate also remained variable. The aims of this study were to characterize twinning in this line and to examine possible association between residual genetic heterozygosity and twinning. This work demonstrates that *O. aureus* gynogenetic parents frequently bear conjoined monozygous twins, and report on a deleterious effect associated with UNH159 that is attenuated in twin embryos.

MATERIALS AND METHODS

FISH

The *O. aureus* gynogenetic line (BIU-1) was produced in the Laboratory of Fish Immunology and Genetics, Bar-Ilan University, by five successive gynogenetic generations, each one followed by full-sib mating (Don & Avtalion, 1988; Avtalion & Don, 1990; Shirak *et al*., 1998, 2002). Gynogens of the fifth generation (F_5) were produced using UV-irradiated milt of *Tilapia zillii* (Gervais 1848) and eggs of full-sib females ($F_4 \times F_4$), as described by Shirak & Avtalion (2001). Their full-sibs ($F_5 \times F_5$) were used in this work, and their twinning rates and types were compared with those obtained in outbred *O. aureus* stock from which the BIU-1 line was originated. Spawns of gynogenetic *O. aureus* were monitored during 5 months of a single spawning season.

FISH REPRODUCTION AND REARING

One year-old gynogenetic and outbred *O. aureus* were divided into four families of one male and five or six females each, and kept in 170 cm × 35 cm × 40 cm aquaria connected to a recirculatory water system thermoregulated to $27 \pm 1^{\circ}$ C (mean ± S.D.). Breeding occurred naturally, under standard conditions. Fertilized eggs were removed from the buccal cavities of brooding females, not later than 4 h after fertilization, and incubated in 750 ml Zuger bottles,

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until complete yolk-sac absorption (8 days after fertilization) at a constant temperature of $28{\cdot}2 \pm 0{\cdot}2^{\circ}$ C. (mean $\pm$ S.D.). Morphological development of embryos was followed up using light microscopy.

TWINS SEPARATION, DNA EXTRACTION AND GENOTYPING

Thirty-nine cases of conjoined twins with common yolk sac were obtained from six successive spawnings of a single selected pair of gynogenetic parents (f1 $\times$ m9) over 5 months. Two days after hatching (5 days after fertilization), conjoined twins were preserved in absolute ethanol. After fixation, they were separated by gentle dissection of the extra-embryonic tissues under a binocular microscope. Forty-four normal full-sibs of the same developmental stage were also fixed in alcohol for comparison in order to examine the possible association between twinning incidences and specific markers. Five of the seven microsatellite markers (UNH101, 148, 159, 187 and 190), which were previously shown to amplify polymorphic products in BIU-1 inbred line (Shirak *et al*., 2006) were polymorphic in both parents and, therefore, were used for the twinning analysis in this study. In order to verify the difference in genotypic segregation for UNH159, additional 84 samples of normal full-sibs were genotyped with this marker. DNA was extracted from twins and from their normal full-sibs by lysis of the whole embryonic tissue following the procedure described by Gates *et al*. (1999).

GENOTYPING OF MICROSATELLITE MARKERS

Polymerase chain reaction (PCR) was applied using Super-Therm Taq DNA polymerase (JMR Holding Inc., London, U.K.), dye-labelled forward or reverse primers and 2 µl of 1:50 diluted lysate. The amplified products were separated on an ABI-377 DNA sequencer (www.appliedbiosystems.com) and automatically sized by comparison with an internal standard using Genescan (version 2.1) (www.nfstc.com) as described by Palti *et al*. (2002).

GENOTYPING OF SNP MARKERS

Genotyping of single nucleotide polymorphism (SNP) markers was performed using DNA MassArray technology (Jurinke *et al*., 2001). External and extension primers were designed by Sequenom assay design software (www.sequenom.com). The matrix-assisted laser desorption-ionization-time of flight (MALDI-TOF) mass spectrometry analysis was applied by a Mass ARRAY genotype analyser (Sequenom).

LINKAGE MAPPING

Three genes, alpha-thalassemia/mental retardation syndrome X-linked (*atrx*), RNA-binding motif protein, X-linked (*rbmx*) and SRY-box containing gene 3 (*sox3*), were used for linkage mapping as they have been previously mapped to linkage group 2 (LG2) near UNH159 (A. Shirak, E. Seroussi, A. Cnaani, T. D. Kocher, G. Hulata & M. Ron, unpubl. data). The expressed sequences orthologous to *atrx, rbmx* and *sox3* genes in *Oreochromis* spp. or other cichlids were detected by basic local alignment search tool (BLAST) in the National Centre for Biotechnology Information (www.ncbi.nlm.nih.gov) and RBEST (http://reprobio.nibb.ac.jp/) databases. By sequence comparison to the relevant *D. rerio* genomic sequences, the exon–intron boundaries were predicted for these cichlid transcripts. Primers (Table I) were designed in adjacent exons using the 'Primer3' programme (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi/). Grandparents and parents of the mapping family (Lee *et al*., 2005) were amplified using BIO-X-ACT Long DNA polymerase kit (Bioline Ltd.; www.bioline.com) according to the manufacturer's instructions and the following conditions: 30 cycles for 30 s at 92° C, 40 s at 63° C and 1 min at 68° C. PCR products were separated on agarose gels, excised from the gel, purified with DNA Montage Gel Extraction Kit (Millipore; www.millipore.com) and then sequenced using an ABI 3700 sequencer. Sequences were assembled and compared using the GAP4 programme (Staden *et al*., 2000).

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TABLE I. *Oreochromis aureus* primers used for marker amplification in *atrx, rbmx* and *sox3* genes

Gene	PT	P	Forward and reverse primers 5′->3′	Extension primer 5′->3′
rbmx	SNP	G/A	GAGGTCCTGGTGGA-ATGAGA AGATGGGGCAGACCTCTTG	ACGTTGGATGTTTAC AAACTGAGCAGACCC
atrx	SNP	C/T	ACGTTGGATGTGTAGCAT-GACTGCAGGAAG ACGTTGGATGCAAGTTCTAC-CATTTTCAC	ACTGCAGGAAGA-GGAAA
sox3	SSR	97/107 bp	FAM-CAGTTGTCTCG-ACTGTGTCCA CCGCACTGTCTTTACATTTCC	NR

atrx, NR, non-relevant; *rbmx*, RNA-binding motif protein, X-linked; SNP, single nucleotide polymorphism; *sox3*, SRY-box containing gene 3; SSR, simple sequence repeat; P, polymorphism; PT, polymorphism type.

One hundred and fifty-six F_2 individuals of the mapping family were used for linkage mapping of the genetic markers. Genotype data for these genes were added to genotype data of the mapping family for >600 markers, and mapping was performed using JoinMap software (3.0) as previously described (Lee *et al*., 2005).

SEQUENCE DATA

Sequence data from this article have been deposited with the European Molecular Biology Laboratory (EMBL) and GenBank Data Libraries under accession numbers: FR821797 and FR821798 for *atrx* of *O. niloticus* and *O. aureus*, respectively; FR733884 and FR733883 for *rbmx* of *O. niloticus* and *O. aureus*, respectively; FR821796 for *sox3* of *O. niloticus*.

STATISTICAL ANALYSIS AND CALCULATIONS

A *t*-test was used for the comparison of twinning rates in spawning of gynogenetic parents and outbred parents. The χ^2-test was used to test differences in frequency of twin types, to compare genotype frequencies between conjoined twins and their normal full-sibs and to analyse distortions from the expected 1:2:1 Mendelian segregation in conjoined twins and their normal full-sibs.

RESULTS

TYPE OF TWINS

Two different categories of twins were distinguished among 152 conjoined twinning cases obtained in 147 spawnings of both outbred and gynogenetic *O. aureus*: (1) twins conjoined by yolk sac only [(Fig. 1(a), (b)] and (2) twins conjoined by both yolk sac and embryonic tissues [(Fig. 1(c)–(e)]. Among the first category, a first type consisting of two normal twins conjoined *via* tissues of the abdominal region [Fig. 1(a)] and a second atrophic type showing clear asymmetry between a normal and a malformed-atrophic twin [Fig. 1(b)] were observed. A few days

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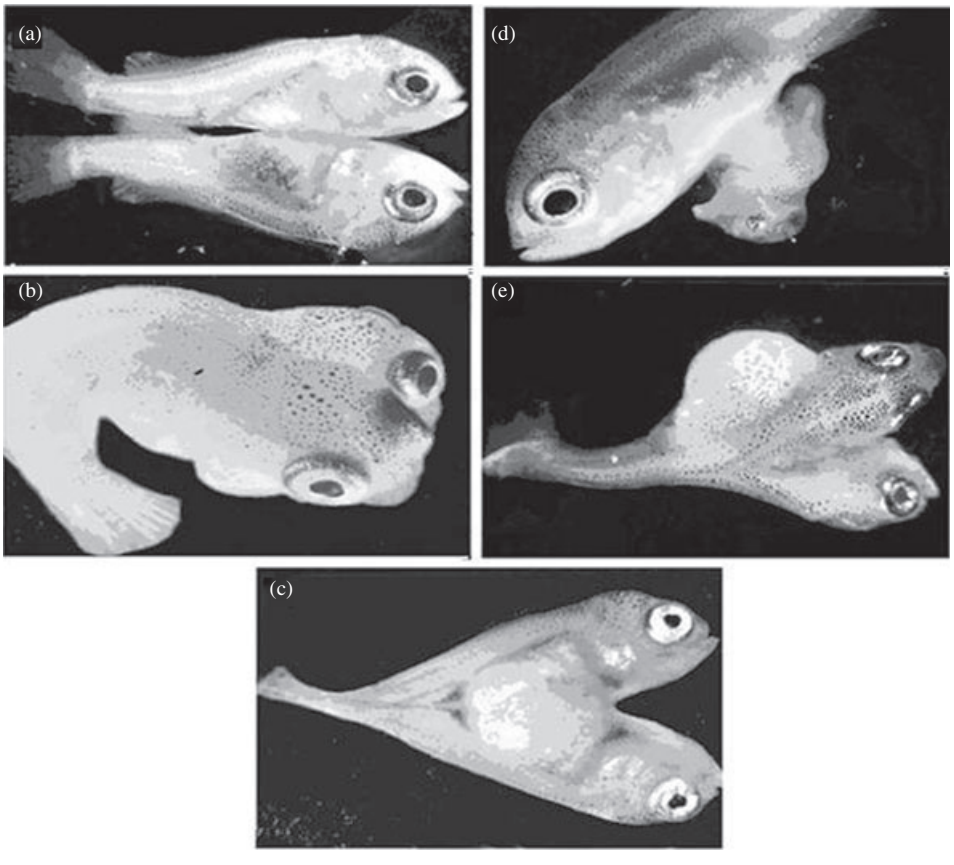

FIG. 1. Conjoined *Oreochromis aureus* twins of different types: (a) normal twins conjoined by tissues of the abdominal region, (b) asymmetric twins (one normal and the other atrophic), (c) cranial duplication with four eyes, (d) bicephalous with latero-caudal thoracic fusion and (e) ventro-caudal fusion.

after hatching, the malformed-atrophic twins developed a haploid-like syndrome of deformations (*e.g.* small size, curved tail, hydropericardium and maldeveloped head and eyes). Within the second category, two twinning types of lateral or ventral conjunctions were observed: (1) conjunctions characterized by antero-lateral cranial duplication with distinguished four eyes [Figs 1(c) and 2] and (2) complete cranio-thoracic duplication resulting in bicephaly [Fig. 1(e)]. The ventrally joined twins showed an abdomino-caudal fusion [Fig. 1(d)]. All twins of these types did not survive beyond yolk-sac resorption.

TWINNING RATES

Average twinning rate in spawn of gynogenetic parents (1·13%) was 12-fold higher than in spawn of outbred parents (0·09%) of the original wild-type stock (Table II). Analysis of twinning rates revealed gradual decrease in twin frequency from early to late spawning in the progeny of four pairs of gynogenetic parents (Fig. 2). Significant differences in frequencies of the twin types were found between spawn of outbred

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TABLE II. Twinning rates in spawn of outbred *Oreochromis aureus* and in offspring of gynogenetic parents

Population	Number of tested spawnings	Mean ± S.D. number of embryos per spawning	Total number of twins	Mean ± S.D. twinning (%)	Range of twins (%)
Outbred	117	312 ± 120	34	0·09 ± 0·08	0–0·9
Gynogenetic	30	348 ± 91	118	1·13 ± 0·60	0–4·1

and gynogenetic parents ($P < 0{\cdot}001$). Yolk-sac-conjoined asymmetric twins were significantly more frequent in offspring of gynogenetic parents than of outbred parents ($P < 0{\cdot}001$). This twinning type was more frequent than other types of twinning in offspring of both outbred (38·2%) and gynogenetic (90·7%) parents (Table III).

ANALYSIS OF UNH MICROSATELLITE MARKERS

To examine the association between twinning and deleterious effects and to test the monozygosity of twins, 39 asymmetric twin pairs were genotyped for five microsatellite markers (UNH101, 148, 159, 187 and 190) and in each pair identical genotypes were observed in both twins (Table IV). Individuals were segregated into homozygotes for the fast migrating allele (F), heterozygotes with both alleles (H), and homozygotes for the slow migrating allele (S). No significant difference in genotypic segregation of these five markers could be found between the asymmetric twins and their 44 normal full-sibs, when each pair of conjoined twins was considered as a single observation for the statistical analysis. Out of the five analysed genetic

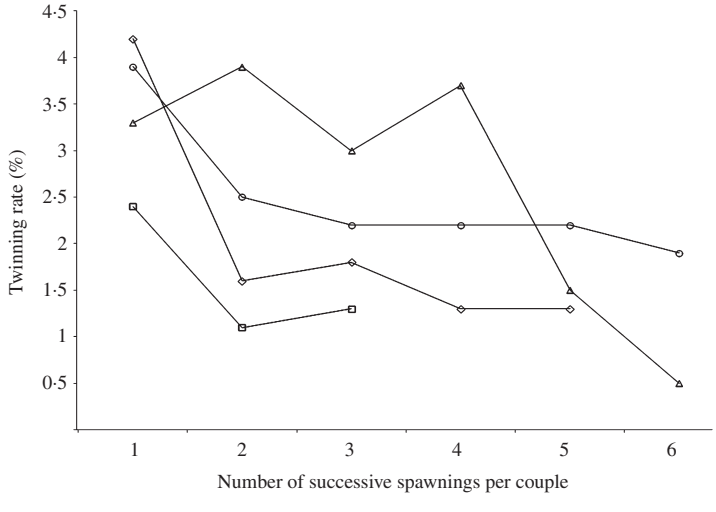

FIG. 2. Gradual decrease of twinning rates from early to late spawning of four gynogenetic *Oreochromis aureus* couples. The twinning rate in up to six successive spawnings of four couples: f8 × m2 (□), f1 × m9 (◇), f12 × m4 (○) and f7 × m18 (△).

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TABLE III. Percentage of different twin types in spawns of outbred and gynogenetic parents of *Oreochromis aureus*. Per cent of twins calculated from total twin cases (in parentheses)

	Common yolk sac only		Conjoining mode	
Parental status	Symmetric	Asymmetric	Lateral fusion	Ventral fusion
Outbred	8 (23·5)	13 (38·2)	11 (32·4)	2 (5·9)
Gynogenetic	3 (2·5)	107 (90·7)	8 (6·8)	0 (0)

markers, only UNH159 revealed skewed Mendelian genotypic segregation in both asymmetric twins and in their normal full-sibs (Table IV). Although the genotypic segregation of UNH159 was not significantly different between twins and their normal full-sibs, F genotypes were only found among twins (Table IV). By including 84 normal full-sibs, a χ^2 analysis indicated a significant difference in genotype frequencies between twins and their normal full-sibs ($P < 0{\cdot}05$; Table V). The ratio between H and S individual types was higher than expected from the Mendelian segregation (2:1) and reached 4·6:1 in normal embryos and 2·3:1 in twins (Table V).

MAPPING OF *ATRX*, *RBMX* AND *SOX3*

The UNH159 has been previously associated with modification of the sex ratio in this line of *O. aureus* (Shirak *et al*., 2002), which may be regarded as analogous to the sex determination function of the mammalian X chromosome. Therefore, this work focused on mapping of three *O. aureus* genes, whose mammalian orthologues are located on the gonosomal region of X chromosome: *atrx*, *rbmx* and *sox3*. The genetic markers were identified as follows.

atrx gene

The human *atrx* protein sequence (BAC81110) was translation (T)BLASTn searched against the RBEST database thus identifying the *O. niloticus* transcript

TABLE IV. Genotypic segregation (F:H:S) for five microsatellite markers in *Oreochromis aureus* asymmetric twins and in their normal full-sibs ($n = 39$ and 44, respectively)

Markers	UNH 101	UNH 148	UNH 159	UNH 187	UNH 190
Twins (pairs)[a]	8:22:9	10:21:8	3:25:11	9:24:6	10:21:8
χ^2_{MS}	0·69	0·44	6·38*	2·54	0·44
Normal full-sibs	9:27:8	10:24:10	0:32:12	10:26:8	12:22:10
χ^2_{MS}	2·32	0·36	15·64**	1·64	0·18
χ^2_{DGF}	0·33	0·12	3·62	0·12	0·13

[a]Genotypic segregation (F:H:S): F, homozygous for fast migrating allele; H, heterozygous; S, homozygous for slow migrating allele; χ^2_{MS}, χ^2 value for goodness of fit to the expected Mendelian segregation (1:2:1); χ^2_{DGF}, χ^2 value for the difference between genotypic segregation in conjoined twins and their normal full-sibs.

*$P < 0{\cdot}05$; **$P < 0{\cdot}01$.

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TABLE V. Genotypic segregation (F:H:S) for UNH159 marker in *Oreochromis aureus* asymmetric twins and in their normal full-sibs ($n = 39$ and 128, respectively)

Genotype	F	H	S	H:S ratio	*P*
Twins (pairs)*	3	25	11	2·3:1	>0·05
Normal full-sibs	0	105	23	4·6:1	<0·001

*F, homozygous for fast migrating allele; H, heterozygous; S, homozygous for slow migrating allele; *P*, value for goodness of fit to expected 2:1 segregation between H and S genotypes.

ONI05GA.39_E01 with high orthology (81% similarity, 63% identity, E-value = 6e-57). To predict exon–intron boundaries of this transcript, it was compared to the orthologous (85% similarity, 74% identity) *D. rerio atrx* gene (ENSDARG00000042236). Sequencing of the third intron (accession numbers FR821797 and FR821798) amplified using PCR primers (Table I) detected the SNP (C/T) that was used for genotyping of F_2 individuals of the mapping family.

rbmx gene

Astatotilapia burtoni (Günther, 1894) mRNA sequence of heterogeneous nuclear ribonucleoprotein G (DQ630739) is orthologous to human *RBMX* (ENSG147274) protein (93% similarity, 92% identity). The *D. rerio rbmx* orthologue (ENSDARG00 000014244, 90% similarity, 84% identity) was used for exon–intron boundary prediction within *A. burtoni* mRNA. Sequencing of the fourth intron (accession numbers FR733884 and FR733883) amplified using PCR primers (Table I) detected the SNP (G/A) that was used for genotyping of F_2 individuals of the mapping family.

sox3 gene

sox3 of *O. niloticus* (DQ632569) is an intronless protein orthologous (98% similarity, 94% identity) to human *SOX3* (ENSG00000134595). As no intronic sequence was available, the gene of 5′-UTR was amplified using PCR primers (Table I). Sequencing of the PCR product (accession number FR821796) revealed a microsatellite (97/107 bp) polymorphism of CA repeats in the mapping family.

Genotypes were unambiguously determined for 85–89 individuals of the F_2 mapping population for genetic markers in these three genes. These genes were mapped on LG2 at positions 27, 30 and 44 cM, respectively, while UNH159 was mapped at 51 cM (Fig. 3).

DISCUSSION

This study used a highly inbred parental line of *O. aureus* that was produced by five successive generations of gynogenesis and full-sib mating after each generation (Shirak *et al*., 2002). The findings associate the higher incidence of conjoined twins with inbreeding as follows: (1) significant 12-fold increase in the conjoined twinning rate as compared to outbred populations which showed gradual decrease from early to late spawns of the same parents, (2) higher proportion of asymmetric twins in gynogenetic offspring and (3) monozygous origin of asymmetric twins. Reduction

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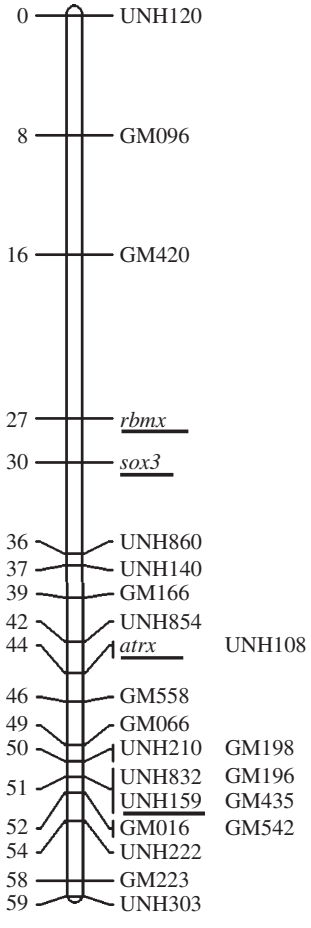

FIG. 3. *Oreochromis* spp. linkage group 2: the mapping positions of UNH159, alpha-thalassemia–mental retardation syndrome X-linked (*atrx*), RNA-binding motif protein, X-linked (*rbmx*) and SRY-box containing gene 3 (*sox3*) (underlined).

in heterozygosity was proposed to be the result of developmental instability in fishes (Leary *et al*., 1985) and in other vertebrates (Naugler & Ludman, 1998). In mice *Mus musculus*, induced parthenogenesis resulted in high rate of conjoined monozygotic twins (Kaufman, 1982, 2004) suggesting that the occurrence of monozygotic twins may be caused by high sensitivity to sub-optimal environment. Furthermore, heat shock applied immediately after fertilization in *O. aureus* and *O. niloticus* increased the frequency of conjoined twins (Owusu-Frimpong & Hargreaves, 2000).

The gradual decrease of twinning rate observed from early to late spawning of gynogenetic *O. aureus* in a spawning season is suggested to be due to a parental ageing effect, as was previously reported by Ferguson (1992) for other traits in rainbow trout *Oncorhynchus mykiss* (Walbaum 1792). In fact, the effect of maternal age on the viability of embryos carrying specific genotypes was previously reported in *M. musculus* (Weichenhan *et al*., 1996) and in the BIU-1 *O. aureus* inbred line (Palti *et al*., 2002). The existence of this maternal effect in humans (Smith & Jones, 1982;

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Boklage, 1987, 1990; Cote & Gyftodimou, 1991) is in line with the above hypothesis. It is possible that older females are provided with higher protection against non-optimal environment. Changes in the gonado-somatic index occurring in fishes and inbred individuals may lead to sub-optimal conditions for gamete development (Heath *et al*., 2002; Gomelsky, 2003; Gallardo *et al*., 2004) and reduction of egg quality in inbred mothers (Bernardo, 1996; Brooks *et al*., 1997; Heath *et al*., 1999). Palti *et al*. (2002) detected 20 markers that remained polymorphic after three generations of gynogenetic inbreeding. Only five of them remained polymorphic in the fifth gynogenetic generation and were used in this work. Significant difference in Mendelian segregation of the UNH159 genotypes was detected between conjoined asymmetric twins and their normal full-sibs. In normal embryos, this deviation consisted of the full elimination of F and partial elimination of S homozygotes, while in conjoined twins the deviation was the result of elimination of the F homozygotes only (Table V). It has been previously shown that genotype-specific elimination at UNH159 locus occurred in normal embryos of this line 2–8 days after fertilization and that the effect was a combination of two deleterious events with distinct onsets: (1) starting at the third day after fertilization leading to the elimination of F homozygotes and (2) starting *c*. 2–3 days later causing the elimination of S homozygotes (Palti *et al*., 2002). Two different explanations to the observed results are likely. First, slower development in conjoined twins was the cause for differences in genotypes' segregation between twins and their normal sibs. Thus, at the age of 5 days, when all embryos were collected, the normal embryos were already exposed to both deleterious effects, whereas the conjoined twins possibly were only exposed to the earlier effect. The second explanation is based on rescue of homozygotes for UNH159 by twinning process through unknown mechanism. The second explanation seems favourable because of the observed weakening of the first deleterious effects in conjoined twins.

Mapping of three *Oreochromis* spp. orthologues (*atrx, rbmx* and *sox3*) located on mammalian X chromosome to the same *Oreochromis* spp. LG2 indicates that some synteny exists between the mammalian X chromosome and *O. aureus* LG2. The mapping position of marker UNH159 on LG2, 7 cM from *atrx*, may signify that this marker is also within this orthologous region. Thus, it is suggested that an unknown genetic factor involved in viability of conjoined twins near UNH159 has an orthologue on mammalian X chromosome.

Comparative analysis of conjoined twins and their normal full-sibs over a longer time span may indicate which of the two explanations is more likely. If slower development in the conjoined twins underlies the attenuation of the deleterious effect, then this attenuation would be diminished.

In conclusion, these results revealed monozygotic origin of asymmetric conjoined twins in a highly inbred *O. aureus* line. A significant increase of twinning rate in the progeny of gynogenetic parents is probably a result of heterozygosity reduction. It is suggested that the region near the UNH159 marker is part of a chromosomal region orthologous to the mammalian X chromosome, which may include the genetic factor that attenuates lethal genotypes in conjoined twins.

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