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Sex Determination Studies in Two Species of Teleost Fish, *Oreochromis niloticus* and *Leporinus elongatus*

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Jean-François Baroiller, Ichiro Nakayama, Fausto Foresti and Daniel Chourrout (1996) Sex determination studies in two species of teleost fish, *Oreochromis niloticus* and *Leporinus elongatus*. *Zoological Studies* **35**(4): 279-285. Genetic analyses of sex determination have identified sex chromosomes in many teleost fish species. However, there are several cases for which sex ratios do not fit perfectly with the expectations of heterogametic systems, suggesting the influence of either minor sex determining genes or environmental influences on the process of sex differentiation. The frequent absence of sex chromosome markers makes the identification of minor sex-determining genes very difficult. It is easier to test first the hypothesis of environmental sex determination (ESD) by studying the temperature effect, since temperature-dependent sex determination has been demonstrated to occur in several vertebrate groups including 1 fish species. To contribute to a better understanding of fish sex determination, we have tested the effects of high temperatures on sex ratios of *Oreochromis niloticus*, and have attempted to isolate sex chromosome molecular markers in *Leporinus elongatus*. Treatments of *O. niloticus* fry at 36 °C applied for 10 days and more, and starting 1 week after fertilization markedly increased the proportion of males, and progeny-testing these males confirmed that some of them are sex-reversed genetic females. Two non-coding sequences of *L. elongatus* Z and W chromosomes were cloned by genomic subtraction. They cross-hybridized with the genome of a close species without providing sex-specific patterns. A collection of *L. elongatus* individuals was subjected to gonadal and chromosomal sexing, and DNA hybridization with both sequences. These analyses revealed 3 individuals having atypical W chromosomes. Interestingly, 2 of these were males having a ZW karyotype. We assume that these atypical sex chromosome arise by exchanges between Z and W chromosomes, and that a transition between female and male heterogamety is underway in this species.

Key words: Fish, Genetics, Sex determination, *Leporinus*, *Oreochromis*.

Although many teleost species are hermaphroditic, the majority of them have separated sexes. The mechanisms of sex determination have been investigated in a number of gonochoristic species, starting from several laboratory models displaying visible sex-linked markers, such as the platyfish, the guppy, and the medaka. Inheritance of these markers indicates the existence of sex chromosomes in these species (Aida 1921). However, cytogenetic studies have so far been unable to confirm their existence. Few teleosts have sex chromosomes which are sufficiently divergent to be recognized on chromosome preparations (Sola et al. 1981, Beçak 1983). Sex determination studies have also been performed in a number of commercial fishes, since farming unisexual populations is generally more profitable than farming bisexual ones. In these species, evidence for a genetic component of sex determination has resulted from observations of sex ratios after sex reversal, gynogenesis, androgenesis, or interspecific hybridization (Hunter and Donaldson 1983,

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Chourrout 1988). An overview of sex determination studies in teleosts reveals that sex ratios seem to be strictly determined by a pair of sex chromosomes in a number of species. This suggests the existence of a major sex-determining gene in the heterogametic sex. The heterogametic sex can be the male or the female depending upon the species, and the evolution from one system to the other has obviously occurred several times during the evolution of teleosts. As a matter of fact, ZW/ZZ and XX-XY species can be found together within the same order, the same genus, and even within the same species (Gordon 1947, Hickling 1960, Kallman 1984). There are also several examples, such as the tilapia mouthbrooders, in which the control by a pair of sex chromosomes seems to be less strict, because the sex ratios deviate more or less slightly from the predictions of heterogametic models (Calhoun and Shelton 1983, Mair et al. 1991). Finally, there are species in which genetic analyses have not revealed the presence of sex chromosomes, such as the Atlantic silverside *Menidia menidia*, whose sex ratio is markedly influenced by temperature level in nature. Investigations of this species in the laboratory have also detected the influence of genetic factors and genotype-temperature interactions on sex ratio (Conover and Kynard 1981, Conover and Heins 1987a,b). So far, *M. menidia* provides the only record of temperature-dependent sex determination in a gonochoristic fish species. But we must admit that environmental influences on the sex ratio have not yet been as thoroughly examined in fish, as genetic effects.

When sex ratios do not strictly match the predictions of the heterogametic system, genetic analysis of sex determination is usually inefficient, because no markers are tightly linked to the major sex-determining gene. Under these circumstances, the recognition and monitoring of minor sex-determining genes in pedigrees are extremely difficult, and may be useless if environmental parameters are entirely or partly responsible for sex ratio fluctuations. For these reasons, we decided to clone sex chromosome markers using as a model *Leporinus elongatus*, one of the rare teleosts having markedly differentiated sex chromosomes (Galetti et al. 1981, Galetti and Foresti 1986). We also studied the effect of variable temperature regimes during sex differentiation on the resulting sex ratio. We used the tilapia mouthbrooder *Oreochromis niloticus* as a model, because its sex imperfectly conforms to an XX/XY sex chromosome system (Jalabert et al. 1974).

MATERIALS AND METHODS

The *Oreochromis niloticus* strain (currently called the "Bouaké strain") originates from the IDESSA fish farm in Ivory Coast. Fry were always collected from natural spawning and removed from mouthbrooding females from 7 to 9 days (post-fertilization) and placed in aquaria for temperature treatment above ambient levels 26-29 °C (applied for all groups before and after the treatment, and for the controls during their entire early life). To measure the effect of temperature, treatments at several temperatures (27 to 36 °C) were applied on 10 full-sib families (produced by 10 single pairs) from 9 to 13 days and for 21 days. Each family was divided into 2-3 groups. To measure the effect of fry age, 4 families (divided into 4-6 groups) were subjected for 21 days to 36 °C treatments starting at different times (7-21 days). To test the effect of treatment duration, 7 families (divided into 3-5 groups) were reared at 36 °C from 9 to 13 days and for 10-60 days. Finally, a single 10-day-old family was divided into 2 aquaria and reared for 21 days at 27 or 36 °C, respectively; a part of each group was sexed after 3 months, while the remainder were raised to maturity. Ten males raised at 27 °C and 15 males treated at 36 °C were individually mated with a single untreated female, and their progenies were raised at 27 °C. In all experiments, sexing was performed after 3 months on more than 100 fish per lot on average, by microscopic examination of gonad squashes (Guerrero and Shelton 1974).

Adult *Leporinus elongatus* used for genomic subtraction were captured from the Tiete River (Brazil). Then, their sex was determined by examining the gonads. Then, the fish were bled for DNA extraction and subjected to chromosome preparation using an air-drying technique on kidney cells after in vitro colchicine and hypotonic treatment (Foresti et al., unpublished results). Other *L. elongatus* were bled to provide genomic DNA for testing the cloned DNA sequences. The sex of these individuals was determined by histology. Two samples of *Leporinus friderici* were also collected in Brazil and French Guyana for the latter purpose. They were sexed by examination of the gonad and bled for DNA extraction. After conventional phenol-chloroform DNA extraction, a single subtraction was performed using a heat-denatured mixture of 1 mg sonicated DNA (average 500 bp) from 8 males and 10 μg of Sau3A restricted DNA from one female, and renatured during 3 days at 68 °C in 200 μl of a rapid re-

naturation buffer (Lamar and Palmer 1984). Then, 3 μg of this solution was subjected to ligation with pUC19 cut by BamHI and dephosphorylated in 20 μl, 4 μl of which was used to transform 200 μl of DH5α Max efficiency *E. coli* strain (Gibco). White colonies which do not express the lacZ gene were screened for the presence of inserts which were purified and tested for sex-specificity by Southern blotting. For this purpose, they were individually labelled with (α-^{32}P)dCTP by random priming and hybridized with restricted genomic DNA from males and females transferred on a Zetaprobe membrane, all under conditions recommended by the manufacturer (Biorad). One sex-specific probe was also labelled with dUTP-digoxigenin (Boehringer Mannheim) and hybridized in situ on metaphase chromosomes using several protocols with slight modifications (Seibl et al. 1990, Zhang et al. 1990). Sex-specific inserts were subcloned in pBluescript KS + for sequencing on both strands by the dideoxy-chain termination procedure (Sanger et al. 1977) using a modified T7 polymerase (Sequenase, USB).

RESULTS

Temperature effects on *O. niloticus* sex ratios

In all experiments, survival rates were not significantly affected by temperature treatments (71.4% versus 76.9% in all controls), which suggests that sex ratios can reflect the results of sex determination.

Temperature levels (Table 1): In almost all families, treatments at 36 °C significantly increased the proportion of males (53% to 81% on average). This effect was less pronounced at 34 °C. Treatment replicates (2/batch) produced similar proportions of males within a given family. Highly significant differences appeared in sex ratios for a given temperature among families.

Treatment stage (Table 2): The male proportion significantly increased for treatments starting not later than 13 days. Those starting between 15 and 21 days led to very moderate effects.

Treatment duration (Table 3): A 10-day-long exposure to 36 °C was sufficient to significantly increase the male proportion, and within each progeny comparable results were obtained with longer treatments.

Progeny-testing of temperature-treated males: Ten temperature-treated males were chosen in a group containing 79% males and individually mated with standard females. Six progeny groups had balanced sex ratios (40%-60%), while the 4 other had a large majority of females (90%-100%). Fifteen males were chosen in the control group containing 46% males and also mated individually with standard females. All the progeny groups had balanced sex ratios (40%-60%).

Table 1. Relation between sex ratios (percentages of males) and temperature levels for treatments starting from 9 to 13 days and lasting for 21 days

	27 °C	28 °C	29 °C	34 °C	35 °C	36 °C
pair 01	43%	—	—	44%	—	61%
pair 02	—	—	44%	66%	—	85%
pair 03	—	46%	—	—	—	90%
pair 04	46%	—	—	—	68%	—
pair 05	49%	—	—	75%	—	90%
pair 06	—	54%	—	—	—	98%
pair 07	57%	—	—	65%	—	76%
pair 08	—	62%	64%	64%	—	—
pair 09	63%	—	—	—	—	84%
pair 10	—	—	71%	—	84%	—

Table 2. Relation between sex ratios (percentages of males) and age at initiation of treatment (36 °C, duration 21 days)

	27 °C	7 days	10 days	13 days	15 days	16 days	19 days	21 days
pair 11	51%	—	93%	86%	—	64%	57%	—
pair 12	52%	—	—	78%	58%	—	—	58%
pair 13	65%	91%	92%	91%	—	77%	—	—
pair 14	72%	99%	99%	94%	—	78%	80%	—

Table 3. Relation between sex ratios (percentage of males) and duration of treatments starting at 9 to 13 days (temperature 36 °C)

	0 days	10 days	20 days	30 days	40 days	50 days	60 days
pair 15	46%	71%	79%	—	—	—	—
pair 16	44%	74%	69%	—	—	—	—
pair 17	49%	—	93%	88%	—	—	88%
pair 18	51%	90%	—	96%	—	—	—
pair 19	51%	88%	87%	87%	84%	—	—
pair 20	45%	81%	80%	84%	—	—	—
pair 21	54%	63%	74%	71%	—	—	—

Cloned sex-specific DNA sequences of *L. elongatus*

Two out of 10 inserts resulting from genomic subtraction provided sex-specific hybridization patterns in Southern blots after restriction with *Hae* III. L'5 insert (729 bp - no homology with any known sequence, no extended open reading frame) clearly hybridized with a sequence present on both sex chromosomes, but with 2 different patterns evoking a restriction fragment length polymorphism. L'46 insert (174 bp - no homology nor extended open reading frame) hybridized at high stringency with a repetitive sequence specific to the W chromosome, and this was confirmed by in situ hybridization on chromosomes (signal on the proximal part of W long arm). Both inserts hybridized with *L. friderici* DNA, but without sex-specific patterns. They did not produce clear signals on rainbow trout DNA. Both inserts were tested on 24 *L. elongatus* individuals of known gonadal and chromosomal sexes (Table 4): 10 phenotypic males having a clear ZZ karyotype provided the typical male hybridization patterns with both probes, and 11 phenotypic females with ZW chromosomes hybridized as typical females. Three atypical individuals were recognized in this study: 1 ZW female having the female hybridization pattern with L'5 but the male one with L'46; 1 male having both male hybridization patterns but an unexpected ZW karyotype, and another male with ZW chromosomes having the female hybridization pattern with L'5 but the male one with L'46. These results were interpreted as 3 possible recombinations between Z and W sex chromosomes (Figure 1) occuring in a region containing the L'5 sequence and the sex-determining locus. This would be located between the long distal W-specific arm and a 2nd W-specific more proximal region containing the L'46 sequences.

Table 4. Compilation of data obtained from chromosome examination and hybridization with the 2 sex-specific probes in 12 phenotypic males and 12 phenotypic females of *Leporinus elongatus*

sex gonad[a] (fish#)	karyotype	L'5 probe[b] *Hae* III	L'46 probe[c] *Hae* III
M1002	ZZ	AB	–
M1020	ZZ	AB	–
M1021	ZZ	AB	–
M1022	ZZ	AB	–
M1029	ZZ	AB	–
M1030	ZZ	AB	–
M1031	ZZ	AB	–
M1216	ZZ	AB	–
M1217	ZZ	AB	–
M1223	ZZ	AB	–
M1026[d]	ZW	ABC	–
M1222[d]	ZW	AB	–
F1001	ZW	ABC	+
F1019	ZW	ABC	+
F1023	ZW	ABC	+
F1024	ZW	ABC	+
F1025	ZW	ABC	+
F1215	ZW	ABC	+
F1218	ZW	ABC	+
F1220	ZW	ABC	+
F1221	ZW	ABC	+
F1224	ZW	ABC	+
F1225	ZW	ABC	+
F1219[d]	ZW	ABC	–

[a]Gonads of animals 1002 to 1031 have been sexed macroscopically, while those of animals 1215 to 1225 have been sexed by histology.
[b]AB is a 2-banded hybridization pattern observed in most males with the L'5 probe, most females providing an additional band (ABC).
[c]Hybridization with L'46 gives a positive (+) signal in most females (3 bands) and no band in all males (–).
[d]Three individuals are classified as atypical from this table (males 1026 and 1222, female 1219).

DISCUSSION

Temperature effects on sex differentiation are clearly demonstrated in this study, since on the one hand male proportions were markedly increased by temperature elevation with a constant survival rate, and on the other hand because 4 out of 10 males resulting from temperature treatment sired a large majority of female offspring, just as genetic females do. It is noteworthy that temperature and sex steroids induce sex reversal during the same stage of development (Hunter and Donaldson 1983). This does not necessarily mean that they act through common mechanisms. The comparison between this study and those on the Atlantic silverside shows that in both cases sex differentiation is dependent upon temperature, genotype, and genotype-temperature interactions (Conover and Kynard 1981, Conover and Heins 1987a,b). A major difference seems to reside in the mode of genetic sex determination that is most likely related in *O. niloticus* to a pair of sex chromosomes, whose existence remains questionable in the Atlantic silverside. Comparison with other vertebrates, such as many reptiles and the amphibian *Pleurodeles waltl* is interesting (Dournon et al. 1990). In the latter species, high

temperatures totally override the normal influence of sex chromosomes, which themselves can entirely determine the sex at basal temperatures. In thermosensitive reptiles, extreme temperatures also can change the sex of all individuals, including those in nature, but the demonstration of genetic sex determination is so far non-existent. It remains to be seen whether temperature influences the sex of *O. niloticus* and *P. waltl* under natural conditions and not only in laboratory. This is clearly the case for thermosensitive reptiles and for the Atlantic silverside, the adaptive function of temperature sex determination being much better understood in the latter case (Conover and Kynard 1981, Conover and Heins 1987b, Bull and Charnov 1989, Conover et al. 1992). Finally, our study clearly suggests that deviations of *O. niloticus* sex ratios from predictions of strict heterogamety might be due to environmental parameters rather than to the effects of minor sex-determining genes. The existence of these genes remains questionable and hard to address because sex chromosome markers are absent so far. This 2nd example of sex thermosensitivity in a teleost fish suggests that such a phenomenon may be more frequent in this group than initially thought. It also encourages the testing of temperature effect in all cases of variable and poorly predictable sex ratios (Streisinger et al. 1981, as an example in the zebrafish). A social control of primary sex differentiation by juvenile size has been recently shown for the Midas cichlid (Francis and Barlow 1993) and could also be tested more systematically. So far, most cases of sex determination by temperature and social environment had been found in sequential hermaphrodite fishes.

The success of cloning sex-specific sequences by genomic subtraction may be explained by the

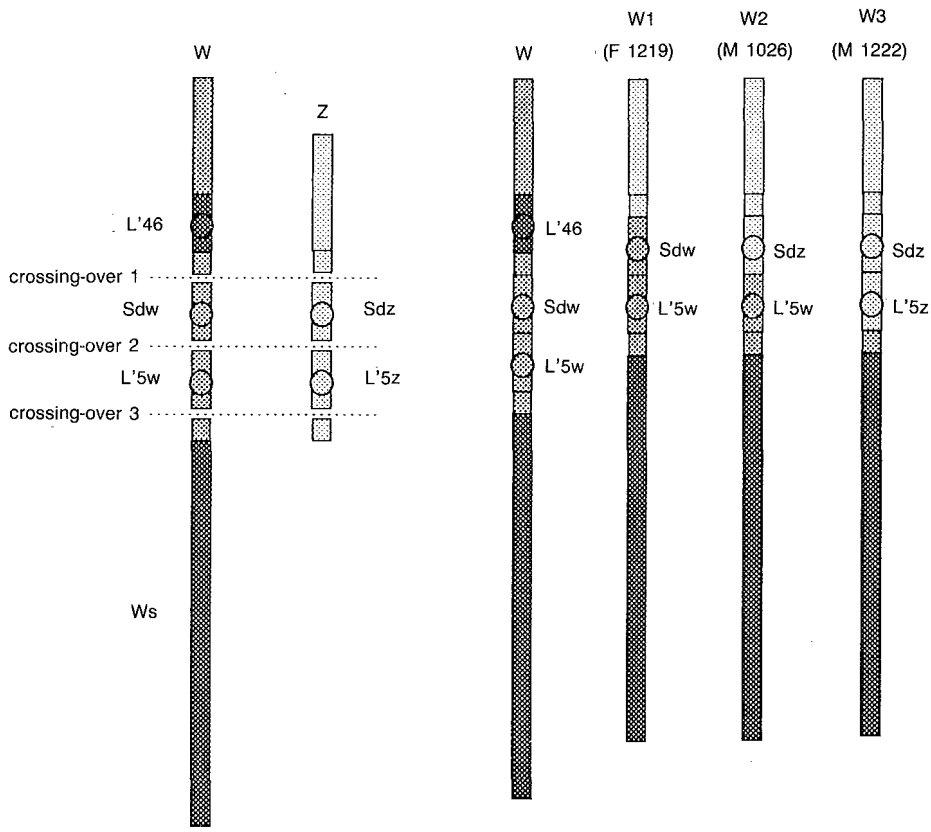

Fig. 1. Hypothetical model explaining the presence of 3 atypical individuals in the collection of *Leporinus elongatus*. Both sex chromosomes contain a pseudo-autosomal region containing the sex determining locus (Sdw or Sdz) and the locus recognized by the L'5 probe (L5w or L'5z), in which crossing-overs take place. Two regions of the W long arm, 1 proximal containing the locus recognized by the L'46 probe and another long and distal determining W morphology (Ws), are located on both sides of this pseudo-autosomal region. Crossing-overs during female meiosis resulted in 3 atypical chromosomes cytologically recognised as W (W1, W2, and W3, belonging to female 1219, males 1026, and 1222 respectively). Another homologous region would contain the euchromatic part of both sex chromosomes.

marked differentiation of *Leporinus elongatus*. However, a Y-linked sequence has been cloned recently by the same method in the chinook salmon (Devlin et al. 1991), in which sex chromosomes have not been recognized cytologically. The latter sequence is not sex-specific in closely-related salmonids, such as the rainbow trout. Both sequences isolated in *L. elongatus* do not mark the sexes of *L. friderici* either, but they cross-hybridize with its genome. All these sequences may therefore result from a recent accumulation of DNA in the genome, directly or indirectly favoring the divergence of sex chromosomes. At least for the L'46 W-specific repetitive sequence, this is in perfect agreement with a "Muller's ratchet"-like process leading to the accumulation of elements where genetic exchange is absent, and thus to morphologically distinct sex chromosomes (Charlesworth 1991). A noteworthy observation in this study is that a minority of individuals have a gonadal sex in apparent contradiction to either the karyotype or the hybridization patterns with the cloned probes. This may be explained by recombinations between both sex chromosomes in their homologous regions. However, the so far unreported occurrence of ZW males is of extreme interest, particularly because they display male hybridization patterns with the cloned probes. This may indicate that the female sex-determining locus has been lost from the W chromosome at a rather high frequency, generating heterogametic males in a heterogametic female species. Such a polymorphism of heterogametic systems is stable in some natural populations of the platyfish (Kallman 1984). It suggests how a transition between XX/XY and ZW/ZZ systems may occur at the level of a species, since all closely related species of *Xiphophorus* have X and Y chromosomes, and that genetic markers suggest that the W chromosome is a derived form. Finally, our study shows how molecular sex-linked markers can detect an unexpected variability of sex chromosomes, which may constitute a partial answer for obscure mechanisms of fish genetic sex determination.

Note: This article includes original results published by Nakayama et al. (1994) Chromosoma 103: 31-39 and by Baroiller et al. (1995) J. Exp. Zool. 273 : 216-223.

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兩種硬骨魚 *Oreochromis niloticus* 及 *Leporinus elongatus* 性別決定之研究

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經遺傳分析好幾種硬骨魚類的性別決定得以鑑定出其性染色體，然而有幾個性比偏離異配系統 (heterogametic system) 期望比的例子顯示出在性別決定的過程中，不是有其它性別決定的次要基因，就是會受到環境因子的影響。由於缺乏性染色體的遺傳標記因子，因此，使得性別決定次要基因的鑑定十分困難。又因爲有若干種脊椎動物 (包括一種爲魚類) 的性別決定具有溫度依存 (temperature-dependent) 的現象，所以，以溫度效應的研究來驗證環境影響性別的假說相形之下較爲容易。爲了瞭解魚類的性別決定，我們測試高溫對 *Oreochromis niloticus* 性比的影響，以及分離 *Leporinus elongatus* 性染色體上的分子標記。以36°C的水溫處理受精後一週的 *O. niloticus* 魚苗十天以上會明顯提高雄魚的比例，由後代分析證實牠們在遺傳組成上是雌性，但是後來發生了性轉變的現象。兩段位於 *L. elongatus* 的Z和W染色體上之非轉譯 (non-coding) 序列經由遺傳組扣除法 (genomic subtraction) 加以選殖 (cloned)，然後將這些序列與一個近緣種的遺傳組作雜結合 (cross-hybridize)，結果並無特定的性別產生 (sex-specific patterns)。一些 *L. elongatus* 的個體先根據其生殖系統及染色體作性別判定，然後以前述兩段序列進行DNA雜結合，結果顯示有三尾魚具非典型的W染色體，而有趣的是其中兩條是具有ZW核型 (karyotype) 的雄魚。因此，我們認爲這些非典型的性染色體是Z和W染色體互換所致，同時這個魚種正由雌性異配轉型爲雄性異配。

關鍵詞：魚，遺傳，性別決定，*Oreochromis*，*Leporinus*。

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